# The C3b Receptor (CR1) on Human Blood Cells

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Abbreviations

| | |
|---|---|
| AHG | Heat Aggregated Human Gamma Globulin |
| BMA | Basal Medium Eagles |
| BSA | Bovine Serum Albumin |
| CIC | Circulating Immune Complexes |
| CPM | Counts Per Minute |
| DABA | Diaminobenzoic Dibrydrochloride |
| DMEM | Dulbeccos' Minimal Essential Medium |
| DMSO | Dimethyl Sulphoxide |
| DNP | Dinitrophenol |
| DRC | Dentritic Reticular Cell |
| E | Erythrocytes |
| EAC | Erythrocytes Sensitized with Antibody and incubated with Serum |
| EDTA | Ethylenediamine Tetra-Acetic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| em | Effective Molecules |
| FCS | Foetal Calf Serum |
| FMLP | N-Formyl-Methionyl-Leucylphenylalanine |
| Fn | Fibronectin |
| gp | Glycoprotein |
| HBSS | Hanks Balanced Salt Solution |
| HEM | Hepes Buffered Earles Minimal Essential Medium |
| HMW | High Molecular Weight |
| HRP | Horseradish Peroxidase |
| IAIH | Immune Adherence Haemagglutination |
| MF | Microfilaments |
| Min | Minute |
| Mol $l^{-1}$ | Moles Per Litre |
| ms | Microspheres |
| MT | Microtubules |
| ND | Not Determined |
| NHS | Normal Human Serum |

| | |
|---|---|
| NMS | Normal Mouse Serum |
| NP-40 | Nonidet |
| NRS | Normal Rabbit Serum |
| OPD | o-Phenylene-Diamine |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| PDBu | Phorbol Dibutyrate |
| PEG | Polyethylene Glycol |
| PHA | Phytohaemagglutinin |
| PMA | Phorbolmyristate Acetate |
| PMN | Polymorphonuclear Leukocytes |
| PMSF | Phenylmethanesulphonyl Fluoride |
| Poly G | Polyguanylic Acid |
| PWM | Poke Weed Mitogen |
| PVP | Polyvinylpyrrolidone |
| RA | Rheumatoid Arthritis |
| RIA | Radioimmunoassay |
| SAS | Saturated Ammonium Sulphate |
| SBTI | Soybean Trypsin Inhibitor |
| SDS | Sodium Dodecyl Sulphate |
| SEM | Standard Error of the Mean |
| SLE | Systemic Lupus Erythematosus |
| TCA | Trichloroacetic Acid |
| TEMED | N N N', N'- Tetramethylenediamine |
| TRITC | Tetramethylrhodamine isothiocyanate |
| Tween | Polyoxyethylenesorbitan Monolaurate |
| VBS | Veronal Buffered Saline |
| V/V | Volume/Volume |
| W/V | Weight/Volume |

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It is the glory of God to conceal
things,
but the glory of kings is to search
things out.

(Proverbs 25:2)

Publications

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Summary

The C3b receptor (CR1) was first isolated from human erythrocyte membranes in 1979 and shown to be a large single chain polypeptide glycoprotein with a molecular weight of 205,000 daltons. CR1 isolated from erythrocyte membranes has been shown in vitro to possess cofactor activity for the I mediated cleavage of C3b to iC3b and C4b to iC4b. It also plays a role in the prevention of lysis of bystander erythrocytes by its ability to cause the decay dissociation of C4b2a3b and C3bBb formed on these cells. In addition erythrocyte CR1 in vivo is thought to play a role in the transport of opsonised immune complexes from the circulation to the reticulo-endothelial system where they can be removed. On unstimulated phagocytic cells the primary function of CR1 is the binding of complexes opsonised with C3 and C4 degradation while on stimulated phagocytes CR1 is able to directly mediate the phagocytosis of opsonised particles. CR1 may play a role in the regulation of B lymphocyte function and on kidney podocytes CR1 may serve to prevent complement activation on the basement membrane of the glomerulus.

The level of erythrocyte CR1 is diminished in patients with systemic lupus erythematosus and at the onset of this study it had not been established if these reduced levels were inherited or if they were acquired as a result of the disease process. One of the main aims of this study was the comparison of CR1 expression on the erythrocytes of monozygotic and dizygotic twins in order to establish the relative roles of genetic and environmental factors on the regulation of erythrocyte CR1 numbers in the normal population.

In order to quantify the number of CR1 sites per erythrocyte CR1 was purified from human erythrocyte membranes and a polyclonal antiserum was prepared. When the monoclonal antibody, E11 became available this was used instead of the polyclonal antiserum as one point determinations could be performed. Using E11 the number of CR1 sites on the erythrocytes of 62 dizygotic and 61 monozygotic twins was calculated. The

CR1 levels on these twins were found to be distributed in a log-normal fashion with no evidence of distinct groups of individuals having high or low phenotypes. Also there was no significant difference in CR1 levels between the sexes nor was there any differences associated with age. Analysis of variance of the $\log_{10}$ transformed CR1 sites/erythrocyte revealed that the intra-pair variance for identical twins was not significantly different from the inter-pair variation between all the twins and the F ratio to compare monozygotic intra-pair variance with dizygotic intra-pair variance was not significant. These results suggest that as the differences between dizygotic twins were no greater than the differences between monozygotic twins, differences in erythrocyte CR1 levels may arise largely or wholly as a result of environmental factors.

As the structural gene for CR1 is present on the gene cluster with C4bp and H, and all three are cofactors for I, the serum levels of C4bp, H and I were measured for each of the twins. It was found that there was no significant genetic influence on the regulation of any of these proteins. However there was a significant increase in the levels of all of these proteins with age. Correlations were found between H and C4bp and between C4bp and I but there were no correlations found between CR1 and any of the other proteins.

Having established that environmental factors play a significant role in the regulation of erythrocyte CR1 numbers in normal individuals, experiments were performed to determine which environmental factors could modulate erythrocyte CR1 expression. Overnight storage of erythrocytes at 4°C or 37°C in RPMI/BSA or autologous serum did not result in a significant reduction in CR1 sites/erythrocyte and storage for two days at 37°C in autologous plasma did not result in an inability to immunoprecipitate CR1 from radiolabelled erythrocytes. Thus it was concluded that spontaneous shedding of CR1 from erythrocytes <u>in vivo</u> was unlikely. High concentrations of the proteolytic enzymes trypsin and elastase removed all CR1 detectable by radioimmunoassay, but at physiological concentrations these enzymes plus thrombin, plasmin, kallikrein and cathepsins C and D had no effect on the number of CR1

sites/erythrocyte. The results of an in vitro and an in vivo experiment suggest that reduction in erythrocyte CR1 numbers could occur as a result of blockade of the receptor by immune complexes. In vitro it was shown that CR1 numbers on the erythrocytes of two individuals were considerably reduced in the presence of large opsonised IgG aggregates as compared with CR1 levels in the presence of large unopsonised aggregates. In vivo numbers of CR1 sites on erythrocytes were reduced in six individuals two hours after drinking 1.2 litres of cows milk which is a rich source of food antigens. These experiments suggest that E CR1 numbers are not a stable heritable characteristic but may be strongly influenced by simple environmental factors.

Having investigated CR1 on erythrocytes attention was then turned to the study of CR1 on polymorphonuclear leukocytes. The number of polymorphonuclear leukocyte CR1 sites/cell was calculated on 14 normal individuals and on 15 patients with systemic lupus erythematosus using the polyclonal anti-CR1 antiserum. It was shown that polymorphonuclear leukocytes express three distinct pools of CR1. CR1 expression was lowest at 0°C (mean 87,593 ± SEM 7,350) but increased when the cells were incubated at 37°C (124,939 ± 16,188) or when the cells were exposed to the chemotactic peptide N-formyl-methionyl-leucylphenylalanine (FMLP) at 37°C (206,591 ± 21,396). The increased expression at 37°C was not dependent upon an intact cytoskeleton or energy. Although the response to FMLP did not require de novo protein synthesis, increased CR1 expression was dependent upon an intact cytoskeleton and energy. Trypsinisation of polymorphonuclear leukocytes removed all CR1 detectable by radioimmunoassay, however after incubation for one hour at 37°C the surface expression of CR1 was restored almost to normal levels. The return of CR1 was not affected by inhibitors of protein synthesis or inhibitors of cytoskeleton assembly. All three polymorphonuclear leukocyte CR1 pools were reduced in patients with active systemic lupus erythematosus, the most severely depleted pool being that which was mobilised in response to chemotactic agents. Polymorphonuclear leukocyte CR1 numbers were normal in patients with inactive systemic lupus erythematosus. Serial studies performed on three systemic lupus

erythematosus patients showed that polymorphonuclear leukocyte CR1 numbers were low during periods of disease activity and increased during remission. These data suggest that low polymorphonuclear leukocyte CR1 numbers in systemic lupus erythematosus are a consequence of the disease.

An attempt was made to study the synthesis of CR1 in cultured monocytes and lymphocytes from normal individuals. The aim of this was to compare the rate of synthesis of CR1 in normal cells with the rate of synthesis of CR1 in the cells from, patients with SLE in order to determine if the reduced CR1 levels associated with the disease were as a direct consequence of the cells inability to synthesise the protein. Although CR1 was successfully immunoprecipitated from one lymphocyte and two monocyte cell cultures, the method did not prove to be sufficiently reliable to be considered suitable for the study of CR1 abnormalities in systemic lupus erythematosus patients.

Clearly environmental factors are important in the reduction of CR1 numbers on the erythrocytes and polymorphonuclear leukocytes of patients with systemic lupus erythematosus. Although immune complexes will reduce CR1 numbers on erythrocytes *in vitro* it is not known if this is the cause *in vivo*. The cause of the reduction of polymorphonuclear leukocyte CR1 in patients with systemic lupus erythematosus is unknown and needs to be resolved. In addition the biological significance of reduced CR1 numbers in polymorphonuclear leukocytes remains to be investigated.

# CHAPTER ONE

## 1.1 History of Complement

As early as 1792 it was noted that blood putrified more slowly than other substances, but it was not until almost a century later that studies on the capability of blood serum to kill certain microorganisms led to the discovery of complement. Following earlier work by Grohmann (1884) and Nuttall (1888), Buchner (1889) showed that bacteria could be killed and lysed by fresh cell free serum and that this activity was destroyed if the serum was heated to 55°C for 30 minutes. He called this activity "alexin", meaning a protective substance, and he considered the killing action to be due to serum proteins with enzymatic activity since it was heat labile and most readily demonstrable at body temperature. Following this, Pfeiffer and Issaeff (1894) showed that the destruction of some microorganisms, for example, Vibrio cholera, required specific antibody as well as alexin. This was a major advance the real significance of which became evident three years later from the work of the Belgian Jules Bordet (1898). Bordet noted that rabbit erythrocytes (E) were dissolved by the addition of horse serum which had been injected previously with rabbit E. If this immune serum was heated to 55°C it lost this activity, but activity could be restored by the addition of serum from an unimmunised guinea pig which had no haemolytic effect on its own. From this Ehrlich and Morgenroth (1900) concluded that there were two substances concerned in the haemolysis reaction: the first, which they called "the immune body", was developed in serum by the process of immunisation and was heat resistant; the second, which was present in fresh serum and was susceptible to heat, they called "complement". Ehrlich went on to show that complement could only act to lyse E when it was linked to the E via the immune body.

Most of the fundamental discoveries concerning complement action were made during the first quarter of this century and sprang from the recognition that complement was a multi-component system. Ferrata (1907) noted that dialysis of fresh guinea pig serum against water yielded a precipitate and a supernatant. On adding the supernatant to sensitised E no haemolysis was observed but if the dissolved precipitate was recombined with the supernatant then haemolysis was restored. Brand (1907) called the factor in the precipitate "midpiece" and the factor in the supernatant "endpiece". These names were given to imply the sequential nature of complement action. The endpiece was found to contain two components called respectively "the second component" and "the third component". The third component was relatively heat stable and could be removed from serum by the action of zymosan or yeast. Serum treated in this way became inactive although it still contained the first and second components. Gordon, Whitehead and Wormal (1926) demonstrated the existence of a fourth component which could be destroyed by treatment with ammonia. Ueno (1938) showed the sequence of action of these four components: E sensitised with antibody first combined with midpiece, now known as activated C1, then with the fourth component C4, and then with endpiece containing C2 and C3.

In 1946 a major advance in the study of complement was made possible by the development of a means to measure quantitatively the extent of haemolysis. This enabled a more detailed study of the conditions which influenced the action of complement. By 1961 Mayer (1961) had demonstrated that the sequence of interaction was C1, C4, C2 and C3 with a requirement for calcium and magnesium. He also introduced the theory that only one lesion per E was necessary for lysis, this was termed "the one hit theory of haemolysis".

Complement research proceeded rapidly: by 1969 C5, C6, C7, C8 and C9 had been purified. By then it was known that complement was a system comprising at least 11 glycoproteins and several inhibitors which could be activated by aggregated immunoglobulins. It was also during the 1960's that the breakdown of the complement proteins C3, C4 and C5 to smaller inflammatory fragments was recognised. With this discovery came the realisation that the complement system not only aided the immunologically specific effects of antibody by the opsonisation and lysis of E and bacteria but also the release of these smaller peptides had effects on the activation of the immune system itself. The study of the role of complement in defence against microbial infections and auto-immune diseases has therefore come to occupy a central position in clinical medicine.

## 1.2 History of the Alternative Pathway

During the early part of this century there was much controversy over the mechanisms involved in the apparent ability of the body to destroy bacteria and viruses despite the fact that it had not been previously challenged with these organisms. While some attempted to explain this natural immunity on the basis that the body had natural antibodies which could destroy foreign organisms immediately on their first encounter, others felt that there was an alternative means of protection which did not involve specific antibody.

In 1954 Pillemer isolated a new serum protein which he believed could be the mediator of natural immunity. This protein was called "properdin" from the Latin pro perdere: to prepare to destroy (Pillemer et al 1954). Pillemer observed that when zymosan particles were added to serum haemolytic complement activity was depleted and the zymosan particles became coated with properdin. This activation step occurred in the absence of antibody and the concentration of the early components C1, C4 and C2 remained unaltered. Thus it was postulated that the properdin system comprised an alternative means of activating the later components without involving the classical pathway. This claim was substantiated by the work of Schur and Becker (1963) which showed that antigen-antibody (Ag/Ab) complexes containing only rabbit $F(ab')_2$ fragments could only turn over complement to a limited extent. To turn over the remainder, the whole antibody molecule was necessary, indicating that there were two kinds of complement in serum, one which bound to $F(ab')_2$ and one which could only bind to the whole molecule.

Sandberg, Oliveira and Osler (1971) studied the ability of the two subclasses of guinea pig IgG to activate complement. $IgG_2$ could utilise all the complement components while $IgG_1$ was only capable of reducing the late components. If $IgG_2$ was digested to yield $F(ab')_2$ fragments then only the late components could be activated. It appeared that there were two different sites on $IgG_2$, one on the Fc portion which could activate the early components and one on the $F(ab')_2$ portion which could activate the terminal components. This second pathway was subsequently found to be activated by microorganisms, endotoxins, certain classes of immunoglobulin and immunoglobulin fragments (Gewurz, Shin and Mergenhagen 1968, Müller-Eberhard and Schreiber 1980).

Further evidence for the existence of an alternative pathway came from a different area of research, the study of snake venom. A factor from snake venom was found to activate the terminal components with little consumption of the early components. (Götze and Müller-Eberhard 1971). Purification of this factor resulted in the ability to analyse more fully the molecular mechanisms of the alternative pathway. It was found that cobra venom factor interacted with two serum proteins termed "factor D" and "factor B" to form a C3 convertase which cleaved C3 and resulted in the activation of the terminal components (Cooper 1973). The knowledge gained from these early studies formed the basis of the current understanding of the alternative pathway which will be reviewed later in this thesis.

## 1.3 Complement

To date recent research has shown that the complement system is comprised of 20 proteins which are for convenience divided into four groups: the classical pathway, the alternative pathway, the terminal sequence and a group of control proteins. The proteins of the classical and alternative pathways form two enzyme cascades which proceed in an orderly sequential fashion similar to the activation of the coagulation and fibrinolytic systems. These enzyme cascades are activated when inactive precursor molecules lose a small fragment through limited proteolysis by a membrane-bound enzyme. The loss of this small fragment reveals a nascent membrane binding site on the remaining larger enzyme precursor which can then bind to the membrane. This membrane-bound enzyme becomes the next functionally active enzyme of the sequence. Each membrane-bound enzyme can activate many substrate molecules so that each step is amplified. The end result of both pathways is the cleavage of C3 which is the central event in the complement system. Following this the proteins of the terminal sequence become assembled into membrane attack complexes which are responsible for the lethal lytic lesions in the lipid bilayers of invading microorganisms. As has been previously described much of the early work on complement focused on the lysis of sheep E in an attempt to define the mechanism of cell lysis. This lysis step was originally thought to be the major role of complement *in vivo* however, in the light of current research the known biological activities of

complement have expanded considerably so that there are now postulated to be at least five vital functions performed by the system (Table 1.1).

## 1.4 Nomenclature

The components of the classical pathway and the terminal sequence are designated numerically in order of their discovery, eg C1, C2, C3, C4, C5, C6, C7, C8, C9. This numbering system can be slightly confusing as the numbers do not necessarily correspond to the order in which the components react. The fragments formed from these native complement components by limited proteolysis are assigned small letters, eg C4a, C4b, C4c, C4d. The alternative pathway components are termed factors and each is represented by a letter, eg factor B, factor D, factor P (properdin). These names can be abbreviated to B, D and P respectively. The control proteins are referred to by their trivial names indicating their functional or historical meaning eg. C1 inhibitor (C1INH), C4 binding protein (C4bp), C3b inactivator (C3bINA) and β1H globulin (β1H), C3bINA and β1H have recently been renamed I and H respectively. The polypeptide chains of each component are suffixed with a Greek letter with α for the largest then β then ɣ, eg C4α, C4β and C4ɣ.

## 1.5 The Classical Complement Pathway

The classical pathway (Fig 1.1) is the name given to the series of plasma proteins which are involved in the assembly of the C3 splitting enzyme, the C3 convertase. It is distinguished from the alternative pathway in that it usually requires the binding of an antigen to its specific antibody for its activation. Once activated a series of reactions occur during which five proteins are transferred from the fluid-phase onto the membrane of the target cell surface. These five proteins are C1q, C1r, C1s, C4 and C2. To ensure that the classical pathway proteins are not activated continuously three control proteins operate, these are C1INH, C4bp and I.

## 1.6 Activation of C1

C1 is the recognition unit of the classical pathway. It consists of three distinct protein molecules, C1q, C1r and C1s (Lepow et al 1963; Gigli, Porter and Sim 1976). These are held together by a calcium dependent bond. C1 circulates in the plasma as a pentamolecular complex containing one molecule of C1q, two molecules of C1r and two molecules of C1s, to give a structural formula of $C1q_1$, $C1r_2$, $C1s_2$.

During activation of the complement pathway C1q is able to recognise and bind to antigen/antibody complexes containing IgM antibodies or the IgG subclasses. $IgG_1$, $IgG_2$ and $IgG_3$ all activate the system with $IgG_3$ being the most potent. However, $IgG_4$ does not activate the pathway and neither do IgA, IgD or IgE. C1q binds to the $C_H2$ domain of IgG and probably to the $C_H4$ domain of IgM, this binding leads to a conformational change in the C1q molecules which subsequently results in the acquisition of enzymatic activity by the C1r and C1s subunits.

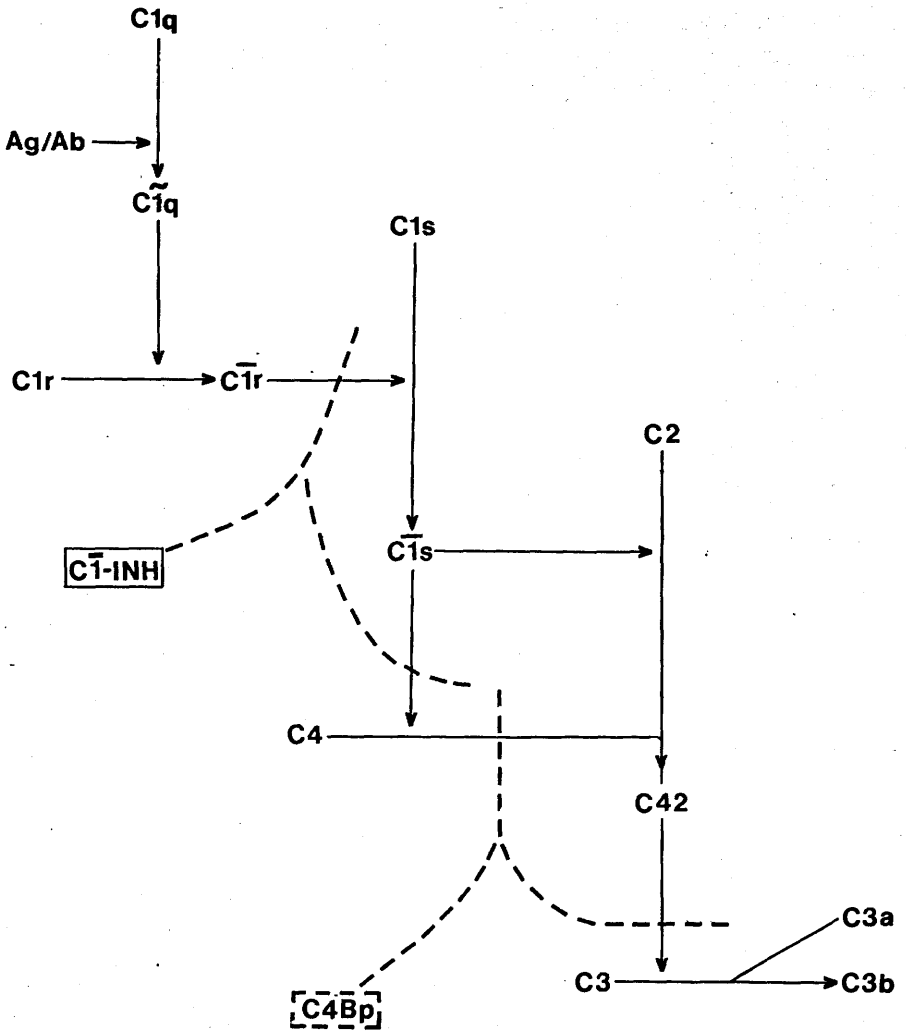
Table 1.1 Biological Activities of Complement

| Activity | Product |
|---|---|
| Pro-inflammatory | |
| 1. Increased vascular permeability | C4a, C3a. C5a, C2-kinin |
| 2. Chemotaxis | C5a, C5a desarg, C5b67, C3bBb |
| 3. Lysosomal enzyme secretion | C3b |
| Cytolytic | C5b-9 |
| Bystander Lysis | C5b-7 |
| Opsonic | |
| 1. Adherence to receptors | C4b, C3b, iC3b, C3d |
| 2. Enhancement of phagocytosis | C3b |
| 3. Increased intracellular killing of bacteria | C3b |
| 4. Increased intracellular degradation of immune complexes | C3b |
| Solubilisation of Complexes | C3b ± C4b |
| Antibody Production | |
| 1. T-dependent responses | C3 |
| 2. Generation of B memory cells | C3 |

Fig 1.1 Classical Pathway Activation

Legend

Diagram showing classical pathway activation. A ~ above a symbol indicates that the component has undergone a conformational change. A bar above a symbol indicates that the component is in its activated state. (From Whaley, K. (1980)).


C1q
Ag/Ab
C1q
C1s
C1r
C1r
C2
C1-INH
C1s
C4
C42
C3a
C3
C3b
C4Bp

C1q has a molecular weight of 410,000 daltons it is formed from 18 peptide chains in three subunits of six. When visualised in the electron microscope each six peptide subunit resembles a bunch of six tulips with the lower half of the stem comprising two pairs of triple peptide helices joined together and the upper half of the stem splitting to form six separate stems, each ending in a globular head (Porter and Reid 1978). The sites for attachment of C1q to the Fc portion of IgG are found in these heads, thus imposing on C1q the potential for multivalent attachment to immune complexes. Indeed for activation of C1, C1q must bind to at least two IgG antibody molecules although one IgM antibody molecule is sufficient (Reid and Porter 1981). The remaining portions of the C1 molecule, C1r and C1s, bind to C1q through C1r which in the presence of calcium ions will bind to the central region of the stalks of the C1q molecule. C1s does not interact with the antibody bound C1q molecule and therefore it is C1r which acts as a bridge between the two molecules. The ultrastructure of both C1r and C1s is now known. They both have molecular weights of 83,000 daltons and are both chemically very similar, each comprised of a single polypeptide chain. Exactly how C1q is able to activate C1s is unknown as C1q has no known enzymatic activity. It is thought that when the heads of the C1q molecule bind to IgG they undergo a conformational change which results in the realignment of the C1r molecule. This realignment of C1r may allow an enzymatic site within the molecule itself to attack one of its own peptide bonds, thus autocatalysing the conversion of inactive C1r to its enzymatically active form, $\overline{C1r}$. $\overline{C1r}$ has the ability to enzymatically activate C1s by cleaving a single peptide in the molecule. Once $\overline{C1s}$ acquires proteolytic enzyme activity it can activate the next two proteins in the sequence C4 and C2 thus causing continuation of the cascade. After formation of C1s the initial recognition phase of the classical pathway is completed and the antibody is no longer required. The control protein C1INH, which is a single chain polypeptide molecule with a molecular weight of 150,000 daltons, binds to the active sites of C1r and C1s. This results in the release of the C1r:C1s:C1INH complex into the fluid-phase where it no longer has any ability to cleave C4 or C2 (Cooper 1985).

## 1.7 Activation of C4 and C2 by C1s

The next phase in the classical pathway is the formation of C4b2a, the classical pathway C3 convertase. This bimolecular complex is formed only after the cleavage of C4 and C2 by $\overline{C1s}$. C4 is composed of three disulphide linked polypeptide chains, an $\alpha$ chain of molecular weight 93,000 daltons, a $\beta$ chain of molecular weight 75,000 daltons and a $\gamma$ chain of molecular weight 33,000 daltons (Schreiber and Müller-Eberhard 1974). $\overline{C1s}$ cleaves a single peptide from the $\alpha$ chain of C4 to produce C4a and a nascent C4b molecule. C4a is released into the fluid-phase while C4b binds covalently through its labile binding site to activating target particles. If C4b does not bind within 50 milliseconds, then the molecule is released into the fluid-phase.

In addition to its labile binding site, C4b possesses two additional stable binding sites. One of these stable binding sites is able to bind to C2 in the presence of magnesium ions to form a C4bC2 complex. C2 has a molecular weight of 100,000 daltons. It binds to C4b, initially in an inactive form, but upon cleavage by $\overline{C1s}$, the fragments C2a and C2b are generated. The enzymatic site is present on C2a the largest of these two fragments (Nagasawa and Stroud 1977). C2a remains bound to C4b to form the C3 convertase C4b2a (Ichihara et al 1986).

1.8 Cleavage of C3 by C4b2a

The substrate for the classical pathway C3 convertase is C3. This protein is functionally the most important component of the complement system. The molecule consists of two polypeptide chains: an $\alpha$ chain of molecular weight 120,000 daltons and a $\beta$ chain of molecular weight 75,000 daltons. These are linked together by disulphide bridges to give a combined molecular weight of 195,000 daltons. Cleavage of the N-terminal end of the $\alpha$ chain of C3 results in the formation of a small peptide C3a (molecular weight 9,000 daltons). C3a is an anaphylotoxin which has a number of biological functions, (i) it directly acts on smooth muscle causing it to contract; (ii) it stimulates mast cells and basophils to release histamine, (iii) it stimulates platelets to release serotonin, (iv) it can induce platelet aggregation and (v) at very high concentrations (50 $\mu$g/ml) it may also be a chemotactic factor. C3a is released into the fluid-phase where it exerts these biological effects for a short while before being inactivated by carboxypeptidase N. The remainder of the $\alpha$ chain and the intact $\beta$ chain comprise C3b (molecular weight 185,000 daltons). C3b is able to bind to target surfaces via its labile binding site which consists of an internal thiolester linking together a cysteinyl and a $\gamma$-glutamyl residue (Law and Levine 1977; Tack et at 1980). Upon cleavage of C3 by the C3 convertase, the labile binding site becomes exposed due to structural rearrangement of the molecule so that the glutamyl residue is available for covalent binding of C3b to hydroxyl or amine groups on the surface of cell membranes or immune complexes. This binding site is available for only a few milliseconds after which the molecule undergoes secondary changes which cause it to lose its binding site. This inactive C3b remains in the fluid-phase where it is unable to participate in formation of a C5 convertase. Approximately 10% of the C3b molecules bind leaving the remaining 90% inactive in the fluid-phase. C3b also possesses a stable binding site which can bind to specific membrane receptors on a variety of cell types, including E, polymorphonuclear leukocytes (PMN), monocytes, macrophages and lymphocytes. Once bound to these cells many important biological functions are elicited. These receptors and their functions are the subject of this thesis and will be discussed more fully in a later section.

## 1.9 Regulation of C4b2a Formation

Since uncontrolled proteolysis of C3 would result in the total depletion of the classical pathway components and consequently an inability to destroy pathogens by this means, regulation of the C3 convertase is vital. For this reason there are a number of control mechanisms.

Internal regulation results from the fact that the labile binding site of C4b is available for a very short period, after which the binding activity is lost. This means that large numbers of C4 molecules must be cleaved to ensure binding of some onto the activating particle. Even when formed, the C4b2a complex is unstable and it decays rapidly by spontaneous dissociation, releasing C2a into the fluid-phase in an inactive form. The natural decay of the enzyme is accelerated by the action of the control protein C4bp (Cooper 1975). C4bp binds to the stable binding site on C4b accelerating the intrinsic decay dissociationof the C3 convertase by displacing C2a from C4b. Recently it has been shown with the aid of monoclonal antibodies that C4b has two stable binding sites. One monoclonal antibody was able to inhibit the binding of both C2 and C4bp to C4b and it could also accelerate the decay dissociation of the C3 convertase. This indicated that the binding sites for C2 and C4bp on C4b are the same or that they partially overlap (Ichihara et al 1986). As well as its ability to decay dissociate the C3 convertase, C4bp can also act as a cofactor for the cleavage of C4b by a third control protein, I (Fujita and Nussenzweig 1978; Gigli, Fujita and Nussenzweig 1979).

I was originally discovered by Nelson (1966) who noted the ability of this protein to abrogate both the haemolytic and immune adherence activity of C3 fixed to sheep E. Fearon (1977) and Pangburn, Schreiber and Müller-Eberhard (1977) isolated I and showed it to be a serum glycoprotein of molecular weight 93,000 daltons which was composed of two non-identical polypeptide chains held together by disulphide bridges. I, together with its cofactor, C4bp, cleaves the $\alpha$ chain of fluid-phase and substrate-bound C4b into the four chain iC4b intermediate (Pangburn et al 1977).

In addition to the cofactor activity of C4bp, the presence of a membrane glycoprotein termed "CR1" can also help to control formation of the C3 convertase (Fearon 1979). This protein was first isolated by Fearon in 1979 and found to have a molecular weight of 205,000 daltons. It was subsequently identified as the C3b receptor responsible for the immune adherence first described by Nelson (1953).

Iida and Nussenzweig (1981) showed that CR1 had the ability to inhibit C3 convertase activity by competing with C2 for the stable binding site on C4b. In this way it could cause acceleration of the decay dissociation of the convertase in an analogous manner to C4bp. In the presence of high concentrations of I and high concentrations of C4b, CR1 can also promote the degradation of C4b to C4c and C4d by I (Medof and Nussenzweig 1984).

This cofactor activity of CR1 is $10^3$-fold greater than C4bp. Additionally, when C3b molecules are also present on the substrate, CR1 is able to mediate the cleavage of C4b even when C4b, CR1 and I are present in low concentrations. Thus before deposition of C3b, C4b is less subject to inactivation (Medof and Nussenzweig 1984).

Apart from the necessity to control C3 convertase formation on target particles there is a further requirement for the protection of the hosts own cells. Accidental formation of convertases on these cells would result in their destruction. A protein has been isolated from the membranes of human E, which has the ability to decay-dissociate C3 convertases formed on E membranes. This protein has been termed "decay accelerating factor" (DAF) and it is proposed that DAF is responsible for intrinsic regulation of convertase formation.

DAF was originally isolated from guinea pig E stroma by butanol extraction (Nicholson-Weller, Burge and Austen 1981) and it had a molecular weight of 65,000 daltons. Although DAF had the same activity as C4bp, antisera raised against DAF did not react with C4bp. These two proteins thus appeared to be distinct. Nicholson-Weller et al (1982) isolated a similar protein from human E and it was found to cause an accelerated decay of C4b2a. DAF was found to be five times more active in decaying the classical pathway convertase than the alternative pathway convertase, whereas the opposite was true of CR1, which decayed the alternative pathway convertase with a ten times greater efficiency than the classical pathway convertase.

Formation of a fluid-phase C3 convertase would result in the undirected activation of the complement system. To prevent this from occuring, fluid-phase C4b can be inactivated by I in the presence of C4bp which is present in high concentrations in the serum, 500 mg/ml. There is now evidence for a soluble form of CR1 which is structurally and functionally related to membrane-bound CR1 and therefore it too may act as a cofactor for the I mediated cleavage of fluid-phase C4b (Yoon and Fearon 1985).

Recently a third group of proteins have been isolated from human leukocytes which may function alongside CR1 and DAF to prevent autologous complement activation. Cole et al (1985) isolated a group of C4b/C3b binding proteins from PMN, lymphocytes and mononuclear cells. These proteins had molecular weights between 45,000 daltons and 70,000 daltons and were thus termed "gp 45-70". They could be isolated by both iC4- and iC3-Sepharose affinity chromatography showing that they have an affinity for both C4b and C3b. These proteins did not appear to be functioning as receptors as polyclonal anti-CR1 inhibited rosette formation between C3b coated E and PMN, monocytes and B lymphocytes. It was therefore postulated that they could be analogous to DAF in that they could bind to C4b and prevent formation of a C3 convertase. Seya, Turner and Atkinson (1986) have also isolated C4b/C3b binding proteins with a molecular weight within the range of 45,000 to 70,000 daltons from human

blood platelets. These proteins are identical to those isolated from human leukocytes and are 50 times more efficient than H in the cofactor I mediated first cleavage of C3b. They also have cofactor activity for the cleavage of C4b but are not as efficient as C4bp. However they do not mediate the decay dissociation of the classical or alternative pathway C3 convertase. These C4b/C3b binding proteins have been re-named "membrane cofactor protein" (MCP).

1.10 Terminal Sequence: Formation of the Classical Pathway C5 Convertase

The terminal sequence is activated when C3 is cleaved by C4b2a, hydrolysing an arginyl serine bond in the N-terminal end of the α chain of C3, resulting in the formation of C3a and C3b. C3b molecules which bind in close proximity to C4b2a on the cell surface change the specificity of this enzyme to one which is capable of cleaving C5. This is termed the C5 convertase C4b2a3b.

C5 consists of a double polypeptide chain with a molecular weight of 185,000 daltons. When C5 binds to the C3b portion of the enzyme it renders it susceptible to proteolysis by C2a, resulting in the production of two fragments, C5a and C5b with molecular weights of 12,000 daltons and 173,000 daltons respectively. C5a is released into the fluid-phase where it exerts a number of effects; (i) it is a potent chemotactic agent, causing neutrophil migration into sites of tissue damage, (ii) it activates neutrophils by triggering the bactericidal oxidative burst, (iii) it switches on neutrophil production of leukotrienes, (iv) it increases vascular permeability, (v) it causes most cell degranulation and (vi) it causes smooth muscle contraction.

The larger fragment, C5b possesses a labile binding site which is exposed only for a few milliseconds. During this time a small percentage of C5b molecules will bind to the cell surface. Both bound and fluid-phase C5b may bind to C6 the next protein in the terminal sequence to form an active C5b-6 complex. This complex then binds to C7 to form C5b67. Fluid-phase C5b67 has a labile binding site which is available for a short while during which it may bind to any lipid layer within its diffusion radius. If it then binds to C8 and C9 cytolysis of adjacent host cells may result. To increase the efficiency with which C5b67 binds to target cells it is thought that these complexes may bind reversibly to fixed C3b molecules scattered over the surface of the cells. Hammer, Abramovitz and Mayer (1976) showed that sheep E bearing rabbit antibody and guinea pig C4b and C3 (EAC4b3) could adsorb C5b6 reversibly. This served to increase the efficiency of the terminal sequence by 100 times over that of cells which had no fixed C3b. In addition, reversible binding meant that C5b could be bound transiently to the C5 convertase, be released and bind to an adjacent C3b molecule leaving the C5 convertase free to cleave another C5 molecule.

C5b6 is a hydrophilic complex, but upon binding to C7 its ability to bind lipid increases. The C5b67 complex becomes firmly attached to the

phospholipid bilayer of the cell membrane where it then binds to C8. This binding initiates the cytolytic reaction which is considerably accelerated by the binding of C9 to C8. The complex which is formed from these five proteins, C5b-C9 is termed the membrane attack complex (Podack, Biersecker and Müller-Eberhard 1978). In a dimeric form the C5b-C9 complex becomes inserted into the lipid bilayer of the cell membrane to form a transmembrane channel. The disruption of the membrane in this manner leads to the breakdown of the cells ability to control the exchange of ions across its membrane and thus eventually to the lysis of E, the killing of nucleated cells, bacteria and the destruction of enveloped viruses (Müller-Eberhard and Schreiber 1980).

## 1.11 Control of C5 Convertase Formation

The last enzymatic step in the complement pathway is the cleavage of C5 to form C5b which then initiates the whole of the terminal sequence. As with the C3 convertase, there are a number of mechanisms which exist to ensure that the C5 convertase does not activate the terminal components in an uncontrolled manner.

Intrinsic control results from the fact that like C4b, the labile binding site of C3b is available for a very short half life of approximately 50 milliseconds. Within this time there is competition between water molecules in the surrounding solution and the protein and carbohydrate moieties on the substrate for the binding site on nascent C3b. This means that only a very small proportion of the C3b molecules will actually bind to the substrate in the vicinity of the C3 convertase to allow formation of a C5 convertase.

C4b is a component of both enzymes and as such it regulates the activity of the C5 convertase in a similar way to its regulation of the C3 convertase. C4bp and DAF bind to C4b and cause the displacement of C2a and thus the active disassembly of the C5 convertase (Iida and Nussenzweig 1981). CR1 has also been shown to be an inhibitor of the C5 convertase. Here it is likely that CR1 competes with C5 for a binding site on the C3b subunit of the enzyme rather than by binding directly to C4b as is the case with the C3 convertase. Fishelson, Schreiber and Müller-Eberhard (1985) showed that addition of 1μg of CR1 inhibited the binding of C5 to zymosan particles bearing C3b by 75%. Addition of purified C5 also blocked the binding of C3b to CR1 of E, thus demonstrating that C5 and CR1 compete for the same site on C3b.

## 1.12 Formation of the Alternative Pathway C3 Convertase

The development of a strain of guinea pigs which were totally deficient in haemolytically active C4 and yet could display normal complement dependent inflammatory responses (Frank et al 1971) confirmed the earlier claim by Pillemer (Pillemer et al 1954) that there was an alternative pathway into the complement system. This pathway was subsequently found to be activated in the absence of specific antibody and to result in the

generation of an alternative pathway C3 convertase which could generate C3 molecules (Fig 1.2). The alternative pathway can be activated by a variety of complexes, which include, polysaccharides, fungi, bacteria, lipopolysaccharides, certain immunoglobulins and some animal cells and parasites. (Götze and Müller-Eberhard 1976). Four serum proteins are required for the generation of the alternative pathway C3 convertase: C3 itself, B, D and P.

Initiation of the pathway is a result of the binding of C3b to B. This binding will only occur in the presence of magnesium ions. The complex which is formed, C3bB, has in itself no enzymatic activity, it requires to be activated by another serum serine protease D which is present in serum in its active form. D cleaves B once it has bound to C3b and produces two fragments: a small one with a molecular weight of 33,00 daltons termed "Ba" and a larger one with a molecular weight of 66,000 daltons termed "Bb" (Lesavre and Müller-Eberhard 1978). This larger fragment contains the active site and it remains in complex with the C3b. It is still uncertain whether Ba is released into the fluid-phase or whether it remains in association with Bb. The resulting enzyme which is formed C3bBb is known as the alternative pathway C3 convertase. D is not incorporated into the enzyme and so it is always available in the fluid-phase to activate more C3bB complexes. D is essential for activation of the alternative pathway in whole serum although serine proteases such as trypsin and plasmin may also be able to cleave C3bB complexes (Brade et al 1976). Formation of the C3 convertase results in the proteolytic cleavage of C3 to C3a and C3b. Thus a positive feedback reaction is set in motion where the product of the enzyme action, C3b, is incorporated into the enzyme itself. This is known as the alternative pathway amplification loop.

At present it is uncertain how the first C3b molecule which activates the whole alternative pathway is generated. There are two main hypothesis. The first is that native C3 may interact in solution at physiological concentrations with B, D and magnesium ions to form the initial C3Bb (Schreiber et al 1978). The C3b molecule produced by this enzyme could then participate in the feedback mechanism. The second is that it may be possible for native C3 to undergo spontaneous hydrolysis as a result of nucleophilic attack on its internal thiolester bond (Pangburn and Müller-Eberhard 1980). The generation of a C3 molecule which is haemolytically inactive but which has properties similar to C3b would enable it to bind to B to form a convertase. This haemolytically inactive C3 has been termed C3($H_2O$), "C3b like C3", C3u or iC3. Certain chemical groups on the surface of cells may facilitate the cleavage of a thiolester bond in C3 and result in the binding of C3($H_2O$) to the cell surface via the active carbonyl group present in the putative thiolester. Like C3b, C3($H_2O$) is cleaved by I in the presence of CR1, gp45-70 or H to iC3($H_2O$) which is similar to iC3b being unable to bind with B to form an alternative pathway C3 convertase thus preventing uncontrolled accumulation of C3bBb on the cells surface.

# The Alternative Pathway

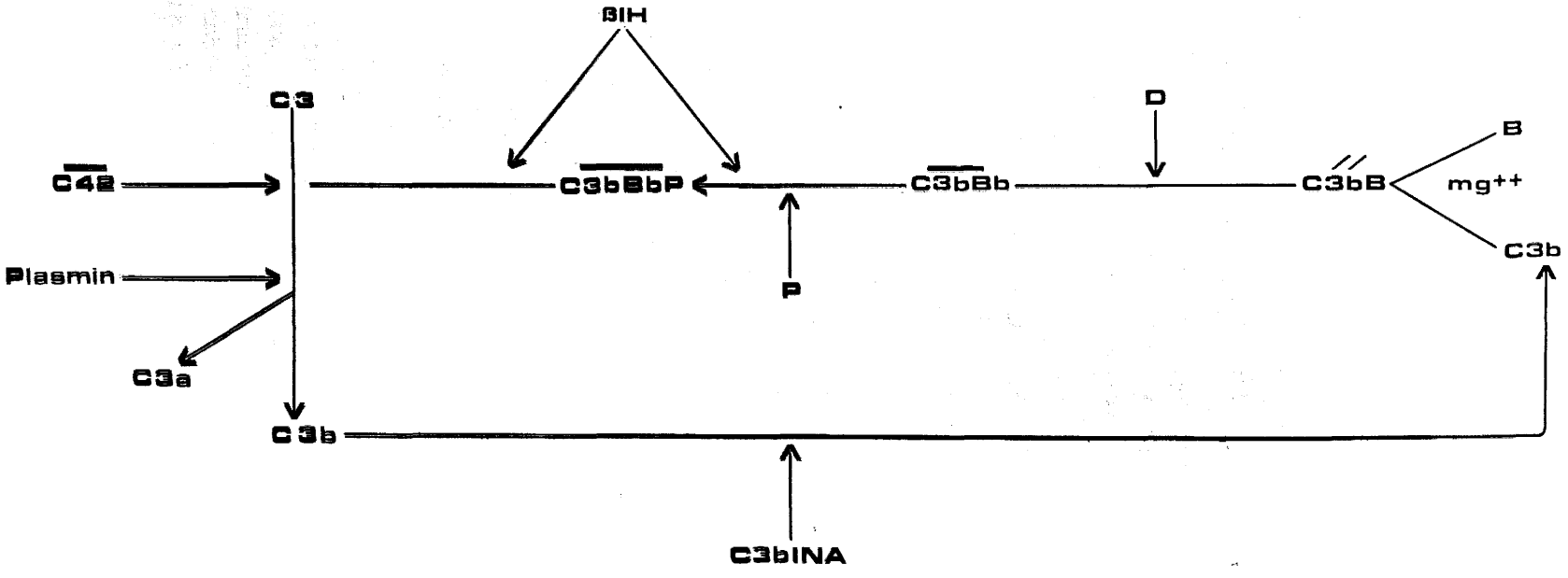
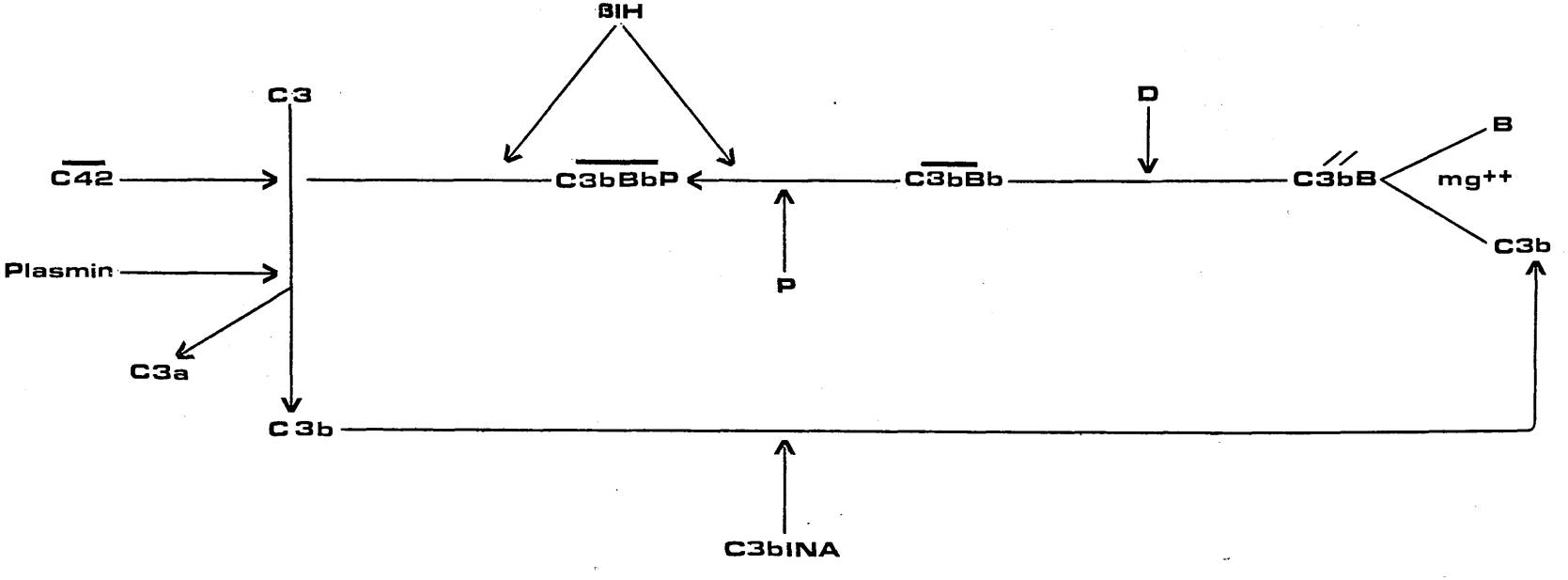


Fig 1.2 Alternative Pathway Activativation

Legend

Diagram showing alternative pathway activation. A // above a symbol indicates that the component has undergone a conformational change. A bar above a symbol indicates that the component is in its activated form. (From Whaley, K. (1985)).

# The Alternative Pathway



## 1.13 Formation of the Alternative Pathway C5 Convertase

A build up of C3b around the C3bBb complex is required to form the C5 convertase (Daha, Fearon and Austen 1976). It was realised that the formation of the C5 convertase required more molecules of C3b than the C3 convertase following experiments which showed that in the fluid-phase combination of C3b, D and B led to very limited lysis of E. However if C3b molecules were fixed to antibody coated sheep E and then the components C3 to C9 were added, the haemolysis reaction was completed with comparable efficiency to that of the classical pathway (Fearon, Austen and Ruddy 1973). Thus the C5 convertase was only formed when the C3 convertase could interact with additional C3b molecules. It is now known that the additional C3b molecules are required since both C5 and B compete for the same or closely adjacent binding sites on C3b. This competition would result in steric inhibition between the two molecules thus preventing formation of a C5 convertase.

The C3/C5 convertase is capable of initiating the cytolytic membrane attack complex, but as with the classical pathway C3 convertase, the alternative pathway C3 convertase is thermodynamically unstable and decays rapidly within two to three minutes due to spontaneous dissociation of Bb. The binding of P stabilises the convertase such that its half life is extended to approximately 30 seconds (Fearon and Austen 1975; Medicus, Götze and Müller-Eberhard 1976). Properdin was first recognised by Pillemer et al (1954) and thought to be an essential component of the alternative pathway. This however is now known not to be the case for, although the activity of the already formed C3 convertase is considerably enhanced by its presence, the alternative pathway can function in the absence of P.

Stabilisation of the convertase can occur by two additional means. In the sera of patients with membrane proliferative glomerulonephritis an autoantibody termed "C3 nephritic factor" (Nef) binds to its antigen C3bBb, reducing the natural rate of decay and increasing the half life of the enzyme. As a result, C3 cleavage is considerably augmented and a severe depletion of C3 occurs in the serum of these patients (Daha, Austen and Fearon 1978).

The second means of stabilisation is by addition of cobra venom factor (CoVF) to serum. Cobra venom contains a factor which produces alternative pathway turnover. Isolation of the factor and the demonstration of its crossreactivity with antibody to human C3 showed that the factor was cobra C3b antibody. Cobra C3b forms a complex with human B thus forming the alternative pathway C3 convertase. As cobra C3b is resistant to the action of H and I the convertase is extremely stable and large amounts of C3 are converted to C3b (Alper and Balavitch 1976; Nagaki et al 1978).

## 1.14 Control of Alternative Pathway Convertase Formation

The ability of C3b to amplify its own production requires that a number of control mechanisms operate to ensure that the system does not become exhausted. This control is mediated by the action of two control proteins, H and I.

H was purified from human plasma and characterised by Whaley and Ruddy (1976) It was shown to be a single polypeptide chain glycoprotein with a molecular weight of 150,000 daltons. Antisera against this glycoprotein reacted with a protein which had previously been known as C3b inactivator accelerator (A C3bINA) because of its ability to enhance the I mediated cleavage of C3b to haemolytically inactive iC3b. From this study it was proposed that H was responsible for this cofactor activity. In this respect it is analogous in function to C4bp of the classical pathway since they both prevent formation of a C3 convertase. In addition to this cofactor activity H has been shown to have two other regulatory actions. It causes decay dissociation of both the C3 and C5 alternative pathway convertase and it can prevent the interaction of C3b with C5 and B thus controlling formation of both classical and alternative pathway C5 convertases. The mechanism by which it exerts this effect is by direct binding to C3b. Evidence for this came from studies which showed the binding of fluorescent and radiolabelled H to C3b coated particles. This binding was in direct proportion to the amount of C3b on the particles and was not observed if the particles were coated with C4 or C2 (Conrad, Carlo and Ruddy 1978). Thus H can bind directly to C3b and displace Bb from the convertase. It can also compete directly with B and C5 for a site on C3b. If H binds in preference to B then no alternative pathway C3 convertase can be formed and if it binds in preference to C5 then no C5 convertase is formed. C3b which has been bound by H is subject to inactivation by I. The iC3b molecule which is formed is unable to bind B. Ross et al (1983) proposed that the B binding site in C3b was probably destroyed totally by cleavage with I as no binding of B to iC3b was detected even in the presence of low ionic strength and nickel cations, conditions which enhance the binding of factor B to C3b. Thus formation of a C3 convertase is inhibited. Whether C3b will bind to H or to B depends primarily on the nature of the surface to which the C3b is fixed. Surfaces which favour the interaction of B with C3b and thus the formation of the alternative pathway C3 convertase are known as "activators". The surface of certain parasites, bacteria, fungi and animal cells are able to activate the alternative pathway by favouring the uptake of B onto C3b (Müller-Eberhard and Schreiber 1980). In this way many microorganisms become coated with large quantities of C3b which eventually leads to their lysis or their ingestion by phagocytic cells. It is possible to convert non-activating surfaces to activating surfaces by chemical modification. This is the case with sheep E which are non-activators. Removal of 80% of their membrane sialic acid results in activation of the alternative pathway and lysis of the cell. The removal of sialic acid from human E does not cause such activation because of the protective function of CR1. As mentioned previously CR1 is a regulator of

the classical pathway C3 convertase, but it was first described as an inhibitor of C3bBb because of its ability to decay dissociate this enzyme and its ability to act as a cofactor for the I mediated cleavage of C3b (Fearon 1979). CR1 thus functions in an analogous manner to H but it differs in that its decay dissociation activity is independent of membrane surface sialic acid. Removal of sialic acid from human E results in an increase in the amount of H required to decay dissociate 50% of the C3bBbP sites on the E whereas the amount of CR1 required remains the same (Fearon 1979). Differences have also been found in the ability of H and CR1 to inactivate the NeF stabilised C3 convertase. Approximately 100 times more H is required to inactive the C3bBbNeF convertase to the same degree as the C3bBb convertase (Weiler et al 1976), while CR1 is five times more efficient at inactivating C3bBbNeF than H (Daha, Kok and Vanes 1982). Thus in patients with NeF, CR1 may serve to protect the host from tissue injury.

DAF causes decay acceleration of the alternative pathway C3 convertase in addition to the classical pathway C3 convertase. In contrast to CR1 which is ten times more efficient in decaying C3bBb than C4b2a, DAF is five times more effective in decaying C4b2a than C3bBb. DAF acts by binding to Bb and causing dissociation of the convertase (Pangburn 1986). In patients with paroxysmal nocturnal haemoglobinuria (PNH), the host red blood cells are unusually sensitive to lysis by complement surviving only six days in comparison to normal E which survive from 80 to 100 days. These patients have an inability to accelerate the decay of C3 convertases which are formed on E due to the random deposition of C3b from the fluid-phase. It was originally proposed that C3 was deposited onto the surfaces of cells from the fluid-phase and that this would result in the continual deposition of low levels of C3 on both host cells and foreign particles alike (Müller-Eberhard, Dalmasso and Calcott 1966). As described earlier spontaneous hydrolysis of C3 results in C3b like C3 which if randomly deposited on cells would be able to bind B and activate the alternative pathway. Normally CR1 and DAF act to protect the E from formation of a C3 convertase but in PNH patients there may be a deficiency of these control proteins. Severely affected PNH-E (type 111 PNH-E) are completely deficient in surface expression of DAF but CR1 from these E appears to be normal as determined by antigenic number, binding affinity, decay accelerating activity and cofactor activity (Roberts et al 1985). From this study it would appear that a deficiency in DAF is a more important contributory factor to the disease than CR1 deficiency. CR1 may be more important in decaying C3 convertases and for inactivation of C3b by I on adjacent surfaces than DAF which may be primarily responsible for regulation of C3 convertases and breakdown of C3b formed on the same cell.

It has recently been reported that CR1 and DAF are restricted from interacting with the alternative pathway C3 convertase to different extents on different cell surfaces. The regulatory activity of DAF towards C3bBb bound to zymosan particles and rabbit E is reduced whereas it is normal when C3bBb is bound to sheep E or bovine E. CR1 has

decreased regulatory activity towards C3bBb on sheep E and on zymosan but not on rabbit E (Pangburn and Tyiska 1985). Thus it may be concluded that the regulatory properties of CR1 and DAF are complex.

1.15 Degradation of C3b

Breakdown of C3b exposes a number of binding sites within C3 for a variety of membrane receptors. The first step in the degradation of C3b is the cleavage by I to iC3b. Pangburn et al (1977) showed that in the fluid-phase I in the presence of its cofactor H could cleave the α chain of C3b. This cleavage resulted in a molecule which had the same molecular weight as intact C3b and was termed inactive C3b (iC3b). The two α chain fragments remained bound to the β chain by means of disulphide bonds. It was later shown that in fact I can produce two cleavages in the α chain resulting in the release of a tiny fragment termed C3f (molecular weight 3,000 daltons) (Harrison and Lachmann 1980). Cleavage of substrate bound C3b by I can proceed in the absence of H (Whaley and Ruddy 1976) although breakdown occurs very slowly. Pangburn et al (1977) showed that dissociation of the α chain from the β chain of iC3b only occured *in vitro* after the addition of tryptic enzymes such as trypsin or elastase. Addition of these enzymes resulted in the production of two fragments termed C3c (molecular weight 14,000 daltons and C3d (molecular weight 30,000 daltons). C3c was released into the fluid-phase leaving C3d bound to the substrate.

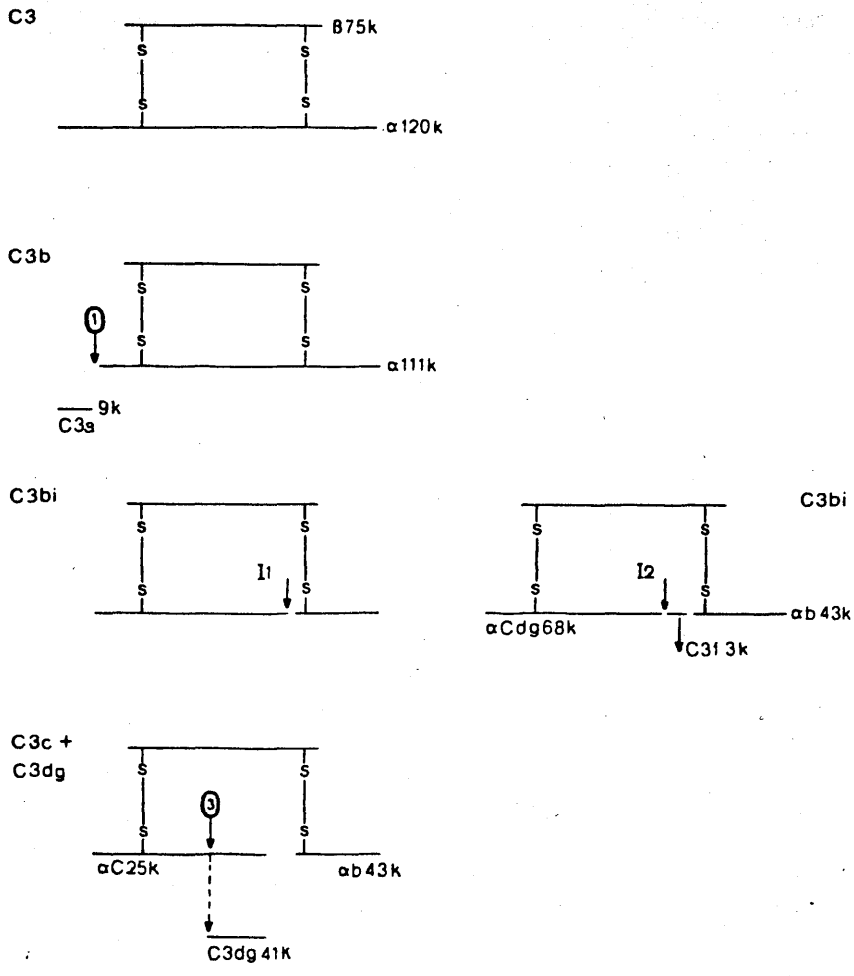
Lachmann, Pangburn and Oldroyd (1982) used monoclonal antibodies to investigate the breakdown of C3. They found one monoclonal antibody which bound to C3c and one which bound to C3d and one which bound to an antigen present in native C3. This last monoclonal antibody was found to be reacting with a fragment termed C3dg. A modified scheme of their proposed breakdown of C3 is shown in Fig 1.3.

Thus iC3b degradation does not result in the immediate formation of C3d, but in the production of C3dg which was previously known as α2D. *In vivo* it is thought that C3dg is not cleaved to C3d and C3g as only C3dg has been detected as the final fragment of complement activation on E (Lachmann 1981; Ross et al 1985). It is also uncertain which of the serum proteases are responsible for the *in vivo* cleavage of iC3b to C3c and C3dg. *In vitro* proteolytic enzymes such as trypsin and plasmin act on iC3b resulting in the generation of C3dg. They may also be responsible for *in vivo* cleavage of iC3b (Lachmann et al 1982). Plasma kallikrein can also cleave iC3b to produce a fragment termed C3d-k (Meuth et al 1983). C3dk is larger than C3d (molecular weight 41,000 daltons) and it was suggested that it could be similar to C3dg and that kallikrein may be the protease responsible for cleavage of iC3b *in vivo*. C3d-k is capable of inhibiting mitogen, antigen and alloantigen induced T lymphocyte proliferation, functions which are not expressed by C3c, C3a or C3b. It is also capable of inducing a two- to three-fold increase in the number of circulating leukocytes when it is intraveneously injected into rabbits. This ability to induce leukocytosis has also been

Fig 1.3 Degradation of C3

Legend

Schematic diagram showing the structure of C3 fragments produced by I proteolysis of substrate-bound C3b fragments. In reaction 1 the α polypeptide chain of C3 is cleaved by a C3 convertase to generate the 9,000 dalton (9K) molecular weight C3a polypeptide and the 186K molecular weight C3b polypeptide. In the presence of H or CR1, I cleaves the α-chain of C3b at two closely spaced sites ($I_1$, and $I_2$). This cleavage releases a 3K molecular weight fragment termed C3f, and generates the three chain C3bi fragment. In reaction 3, I in the presence of H or CR1 cleaves the α C3dg chain of C3bi, to release the C3c fragment into the fluid phase leaving the C3dg fragment bound to the substrate. Although the 8K molecular weight C3g fragment may be cleaved from the C3dg fragment by various proteases forming fluid-phase C3g and substrate-bound C3d (33K), it is not thought that this final breakdown occurs in the blood.


C3
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S
S
S
S
α120k
C3b
S
S
S
S
α111k
9k
C3g
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S
S
S
I1
C3bi
S
S
S
S
I2
αb43k
αCdg68k
C3f 3k
C3c +
C3dg
S
S
S
S
αC25k
αb43k
C3dg 41K

attributed to a fragment termed C3e which is presumed to be cleaved from the α chain of C3c by an as yet unknown proteolytic reaction. This fragment was isolated by Ghebrehiwet and Müller-Eberhard (1979), it had a molecular weight between 10,000 and 12,000 daltons and it could induce a two- to three-fold increase in the number of circulating leukocytes in rabbits. Meuth et al (1983) concluded that as C3d-k and C3e could both induce leukocytosis that C3d-k contained both the C3e and C3d portions.

### 1.16 The Role of H, C4bp and CR1 in the Proteolytic Breakdown of C3b

As previously mentioned H is an essential cofactor for the I mediated cleavage of C3b in the fluid-phase, being more important for this cleavage than C4bp. Fujita and Nussenzweig (1979) showed that on a weight basis H was 20 times more efficient than C4bp in acting as a cofactor for the cleavage of C3b in solution. In the case of cell-bound C3b, although I can function in the absence of H, the presence of H can potentiate the cleavage of C3b by approximately 30 fold (Whaley and Ruddy 1976). C4bp is without effect even at 300 times the amount of H on a weight basis (Fujita and Nussenzweig 1979)). Therefore it is likely that the main function of C4bp is directed towards cleavage of C4b and not C3b.

CR1 has been reported to be $10^4$ to $10^5$ times more effective on a weight basis than H in acting as a cofactor for the I mediated conversion of substrate-bound C3b to iC3b (Medof et al 1982; Medof and Nussenzweig 1983). It has also been proposed that under physiological conditions CR1 acts as a cofactor for the further cleavage of iC3b to C3c and C3d (Medicus, Melamed and Arnaout 1982; Medof et al 1982; Ross et al 1982). Medicus et al (1982) reported that in the presence of CR1, I could cleave iC3b bound to human or sheep E to release C3c into the fluid-phase. In the presence of I depleted serum no other serum enzyme was capable of cleaving iC3b bound to human E. Thus it appeared that CR1 and not H was responsible for the further breakdown of iC3b. In vivo this suggested that one of the functions of CR1 was to allow breakdown of iC3b to smaller degradation fragments thus avoiding an inappropriate inflammatory response caused by biologically active fragments of C3b. These conclusions were supported by the findings of Ross et al (1982) who observed that sheep E coated with C3b and incubated with isotonic heat inactivated serum for four hours at 37°C had little or no bound C3dg. If however the EC3b were treated in the same serum in the presence of 10μg/ml of purified CR1 for 1 hour at 37°C, 50% of the bound iC3b was cleaved into bound C3dg. In the presence of low ionic strength and heat inactivated serum, 95% of the bound iC3b was converted to C3dg, indicating that under low ionic strength conditions H can act as a cofactor for the cleavage of iC3b.This suggests that under physiological serum concentrations in whole blood that it is CR1 rather than H which is the cofactor for I.

Medof et al (1982) investigated a previous observation which had shown that when soluble immune complexes are added to a mixture of normal

human serum and autologous unseparated whole blood cells they fix complement and become bound to E via C3 fragments (Medof and Oger 1982). This binding could not be prevented by preincubation of the complexes with I and H, indicating that E could bind iC3b containing complexes. Subsequent addition of I alone to the E resulted in the release of the complexes and the concomitant release of C3c indicating that E CR1 was capable of cleaving membrane-bound iC3b to C3dg and C3c in the presence of I. These findings have been interpreted as meaning that processing of C3b containing immune complexes in vivo occurs on the surface of red cells. This function of E CR1 will be discussed more fully in a later section.

Medof and Nussenzweig (1984) have reported that the presence of C4b molecules can enhance the CR1 cofactor I mediated cleavage of iC3b. This enhancement can be inhibited by addition of anti-C4b antiserum or C2a thus indicating that C4b molecules are responsible. This effect was explained by postulating that C4b acts to promote the interaction of iC3b with CR1. CR1 may be present on the membrane of PMN and monocytes in discrete clusters (Petty et al 1980; Abrahamson and Fearon 1983). Clusters of C4b and C3b could serve as more effective ligands for CR1 allowing formation of multiple bonds between ligand and CR1. This in turn may explain why CR1 is a more effective cofactor for I than H on substrate-bound C3b. Medof and Nussenzweig (1983) showed that in the fluid-phase H and CR1 support the cleavage of C3b to iC3b with comparable efficiency. However CR1 was $10^3$ times more effective than H in the cleavage of substrate-bound C3b and this efficiency increased as the number of C3b molecules increased due to clustering around the C5 convertase. Thus in vivo it may be that CR1 is capable of inactivating C3b found clustered around the C5 convertase whereas H is able to bind to and inactivate unclustered fluid-phase C3b molecules.

Although it is generally accepted that the breakdown of surface-bound iC3b is mediated by I plus CR1, recent studies by Malhotra and Sim (1984) and Jepsen et al (1986) suggest that breakdown could be mediated not by I plus CR1 but by leukocyte proteases which may be present in the experimental systems used due to the contamination of E by leukocytes. Evidence also exists to suggest that the cleavage of soluble iC3b is mediated by proteases. Sim and Sim (1983) could not demonstrate the breakdown of purified soluble iC3b in the presence of I and CR1, and Yoon and Fearon (1985) found that the breakdown of soluble iC3b in the presence of I required a concentration of CR1 which was two orders of magnitude higher than the concentration of the receptor which was detectable in the plasma.

## 1.17 Introduction to Complement Receptors

As can be seen from the preceding account, the receptor for C3b, CR1, plays an important role in many of the functions of the complement system. However, although researchers have been aware of the existence of such a receptor for more than 30 years it is only within the past ten

years that significant advances have been made in understanding its properties and functions. The remainder of this introduction will address itself specifically to CR1 and to the additional receptors which have been discovered for the further proteolytic cleavage products of C3b namely CR2, CR3 and CR4.

## 1.18 History of Complement Receptors

In 1953 Nelson (1953) observed that treponemes isolated from testicular syphilomas of rabbits would in the presence of antiserum from syphilitic patients adhere to normal human E. This adherence was found to be dependent on the presence of a specific antibody and some component of normal human serum which was thought to be complement. Nelson termed this immunologically specific reaction immune adherence. Twenty three years before this Duke and Wallace (1930) had described an adhesion phenomenon in which, under certain conditions, red blood cells from a monkey adhered to Treponema pallidium in the presence of specific trypanosomal antibody. They called this "red cell adhesion". In 1959 Nelson and Nelson (1959) characterised the nature of this adhesion bond and found it to be temperature sensitive, destroyed by trypsin treatment, and stable at high salt concentrations and over a wide pH range. They therefore suggested that as adhesion was destroyed by trypsin a receptor may have been responsible for the bond. In the years which followed attempts were made to discover which components of complement were responsible for immune adherence. Nishioka and Linscott (1963) noted that although guinea pig and human complement were equally reactive in immune adherence the guinea pig serum was more haemolytically reactive than the human serum. This implied that there was a difference in the participation of complement in immune adherence and in haemolysis. They postulated that a complex intermediate step between the first three reactive complement components and the final lysed E may be responsible for immune adherence. By preparing sheep E sensitised with antibody against the Forssman antigen on E and then coated with C1, C4 and C2 (EAC142) they were able to demonstrate that no immune adherence occurred. However if C3 was added to the EAC142 the E became reactive in immune adherence. The work of Nelson (1963) confirmed these results and also provided evidence that complement could function as an opsonising agent. When EAC1423 were mixed with leukocytes from a guinea pig there was a marked increase in the rate and degree of phagocytosis of the complex as compared to phagocytosis when C3 was absent from the complex. A year later Gigli and Nelson (1968) provided further support for the theory that C3 was the ligand responsible for both immune adherence and opsonisation of immune aggregates. They showed that C4 and C3 were essential for immune adherence and immune phagocytosis and that neither the removal of C1 and C2 from the complement coated E nor the addition of C5, C6, C7 or C8 changed their reactivity in these reactions.

It therefore seemed possible that receptors on leukocytes which reacted specifically with C3 bound to the surface of E or immune complexes could promote phagocytosis and immune adherence.

Lay and Nussenzweig (1968) presented evidence for the existence of such a C3 receptor. While investigating the binding of mouse leukocytes to sheep E it was found that the leukocytes would cluster around E which had been sensitised with antibody and then incubated in fresh normal mouse serum (EAC). These clusters were called rosettes. This was the first semi-quantitative method for evaluating the interaction of receptors and ligands on various cell surfaces. Using this technique it was found that E sensitised with IgG antibody (EAIgG) formed spontaneous rosettes with mouse peritoneal cells but not with blood lymphocytes; further incubation of EAIgG with serum caused a large increase in the percentage of rosettes formed on blood lymphocytes, monocytes and PMN. However, E sensitised with IgM antibody (EAIgM) did not form rosettes with any cells unless they had first been incubated in fresh mouse serum whereupon they rosetted with peritoneal cells and some blood leukocytes. These results indicated that there were at least two different types of receptor involved in adhesion of leukocytes to E. One which involved IgG and which has become known as the Fc receptor and one which involved some component of complement. Huber et al (1968) reported that human monocytes also had distinct receptors for complement and IgG. Monocytes bound to and ingested EAIgG; this binding was inhibited by the addition of fluid-phase IgG, but binding could be restored by addition of C1, C2, C4 and C3. Thus he proposed that these two receptors cooperated in attachment and ingestion of particles. Bianco, Patrick and Nussenzweig (1970) showed that B lymphocytes had a receptor for complement. It was noted that T lymphocytes did not bind to EAC complexes whereas B lymphocytes did and it was proposed that binding to EAC could be used as a marker to distinguish between the two populations. The B lymphocytes were called complement rosetting lymphocytes (CRL). C3 was necessary for this rosetting since, (i) EAC prepared with C4 alone did not bind, (ii) depletion of C3 by CoVF also destroyed rosetting and (iii) addition of papain F(ab) fragments of rabbit anti-mouse C3 inhibited rosetting (Eden, Bianco and Nussenzweig 1971). As C3 on EAC1423 cleaved to C3b it was proposed that C3b was the ligand involved in the binding of EAC to CRL. It seemed possible that there could be an intimate relationship between the immune adherence receptor on human B lymphocytes, monocytes and PMN since both require the presence of C3 or its breakdown product C3b.

Cooper (1969) demonstrated that the immune adherence receptor on E could also bind C4b as well as C3b. It was not then known if there was a separate receptor for C4b or if the same receptor for C3b was recognising a common reactive group on both proteins. However, Ross and Polley (1975) showed that human E, B lymphocytes, monocytes and PMN all had a receptor activity for C4b and for the C3c region of C3b thus demonstrating that the immune adherence receptor of these cells was specific for both C4b and C3b.

The existence of a second complement receptor on lymphocytes was discovered following a consideration of questions raised by a study to determine if lymphocytes from patients with chronic lymphatic leukemia

(CLL) resembled more closely B or T cells (Ross et al 1973a). This study showed that the leukemic cells reacted poorly with human C3 as compared with mouse C3. EAC mouse (EACm) could detect up to 30-fold more CRL than EAC human (EAChu). Since this finding was in marked contrast to normal CRL which reacted more strongly with EAChu, Ross proceeded to investigate why this should be so (Ross et al 1973b). It was found that EACm which had been prepared with C3b deficient mouse serum contained two forms of C3, C3b and a product of C3b cleavage C3d while EAChu which had been prepared with purified complement components contained only C3b. Thus it was proposed that a second complement receptor for C3d existed. The discovery of the C3d receptor made it possible to understand the results of the first study (Ross et al 1973a). Leukemic lymphocytes reacted poorly with EAChu because they lacked the C3b receptor but they reacted strongly with EACm indicating that they possessed a C3d receptor. This C3d receptor on leukemic lymphocytes was antigenically related to a receptor found on normal B lymphocytes as antisera to leukemic cells could inhibit rosettes between normal lymphocytes and EACm.

Confirmation of the existence of a second complement receptor for C3d was provided by Eden, Miller and Nussenzweig (1973) who demonstrated that normal B cells could bind to EAC3b and EAC3d, while granulocytes could only bind to EAC3b. It was concluded that human B cells possessed a complement receptor for C3d and also one for C3b while granulocytes only possessed one for C3b.

Reynolds et al (1975) reported the existence of a C3d receptor on human monocytes and alveolar macrophages. However this receptor was only detected when EA were reacted sequentially with individual complement components to form EAC3d and not when fresh human serum was used as the complement source. Later work revealed that the C3 used to prepare EAC3d was contaminated with traces of H and I which had resulted in the conversion of C3b to iC3b (Pangburn et al 1977) and not to C3d as was originally thought. EAC3d complexes prepared with whole serum did contain C3d as the presence of serum enzymes allowed the further cleavage of iC3b to C3d. Therefore it seemed possible that monocytes and macrophages could in fact be binding to a third stable binding site for iC3b.

Ross and Rabellino (1979) tested a variety of cells with EAC3d prepared with H and I and enzymes and EAC3bi prepared with H and I alone. They found that PMN and monocytes were unreactive with EAC3d whereas variable proportions of these cells formed rosettes with EAC3bi. These iC3b rosettes could not be inhibited with fluid-phase C3c or C3d fragments which inhibit the C3b receptor and the C3d receptor respectively. Thus PMN and monocytes were proposed to possess a receptor for iC3b which was distinct from the receptor for C3d and C3b.

Using fluorescent microspheres (ms) coated with iC3b, Ross and Lambris (1982) reported that, E, PMN, monocytes and a proportion of lymphocytes

bound to these ms. This binding on nonlymphoid cells could not be inhibited by fluid-phase C3b, C3c or C3d or antiserum against the C3b and C3d receptors. On B cells the situation was more complex as binding of iC3b to the C3d receptor occurred by way of the d region of the intact iC3b molecule. A small population of B cells were found to rosette with iC3bms even when all the C3d receptor sites were blocked with fluid-phase C3d. This subset of B cells were separated on a fluorescent activated cell sorter and were found to contain all the natural killer (NK) activity in the human blood (Ault and Springer 1981). These NK cells also reacted with the monoclonal antibody M170 directed against the iC3b receptor (Beller, Springer and Schreiber 1982).

The realisation that specific fragments of C3 could have a binding site for more than one ligand meant that the distribution of the receptor types on different cells required reappraisal. Ross et al (1983) demonstrated that iC3b could bind to all three of the complement receptors but with differing affinities. Fluorescent ms coated with iC3b bound to E in the presence of ethylenediamine tetra-acetic acid (EDTA), which inhibited binding to the iC3b receptor. The addition of fluid-phase C3b or iC3b inhibited this binding and indicated that E possessed only one receptor for C3b which could also bind iC3b. The binding of iC3b to E was weak and required ten-fold more iC3b molecules than C3b molecules to achieve the same level of adherence. In addition, iC3b binding only occured at low ionic strengths and not at physiological salt concentrations. Microspheres coated with iC3b were also found to rosette with Raji cells which bear only the C3d receptor. This result confirmed the earlier work of Ross and Lambris (1982) which had proposed that lymphocytes could bind iC3b complexes to the C3d receptor by way of the d region of the molecule.

Monocytes and PMN were also studied for their ability to rosette with EC3d, C3dms, EC3dg and C3dgms. It was found, in agreement with other studies, that these phagocytic cells did not bind to C3d, having no C3d receptor. They did however bind C3dgms and EC3dg. This binding occurred in the presence of EDTA and anti-CR1 antisera indicating that C3dg was not binding to the iC3b receptor or to the C3b receptor. Addition of soluble C3d sheep E membrane complexes (C3d-OR) did inhibit rosettes. This suggested two possible explanations. First, it was proposed that phagocytic cells express C3d receptors but in very low numbers detectable only with C3dg which has a higher affinity for the C3d receptor than C3d. Secondly, it was suggested that these cells may possess a fourth receptor similar in ligand specificity but different in structure from the C3d receptor of lymphocytes. In support of this second theory Frade et al (1985a) showed that neutrophils could form rosettes with E bearing large amounts of C3dg. This rosetting could not be inhibited by the addition of excess anti-C3d. From this observation the receptor on phagocytic cells has tentatively been described as the fourth complement receptor.

Since the receptors had originally been named according to the ligand to which they bound the discovery of their ability to bind to more than one ligand threatened terminological confusion. It was therefore decided to rename the receptors according to their order of discovery. Accordingly, the nomenclature is now CR1, the original immune adherence C3b receptor, CR2, the C3d receptor, CR3, the iC3b receptor and CR4, the C3dg receptor. Table 1.2 lists the specificity and cellular distribution of these four receptors.

## 1.19 Isolation and Structure of CR1

Most recent research on complement receptors was made possible by the ability to isolate the receptors thus allowing a more detailed study of their structure and function.

Following the observation that desialated human E were protected from lysis in normal human serum, Fearon (1979) attempted to discover if a regulatory protein was responsible for this. He succeeded in isolating a glycoprotein (gp) of molecular weight 205,000 daltons from human E. This protein was found to accelerate the decay of the properdin stabilised amplification C3 convertase, C3BbP, by displacing Bb from C3b. It could also promote cleavage of C3b by I. Attempts to identify the protein showed that anti-gp 205 could block in a dose dependent manner the ability of human E, PMN monocytes and B cells to rosette with sheep EAC3b (Fearon 1980). This inhibitory effect of anti-gp 205 was specific for C3b as it did not inhibit rosettes between monocytes and EiC3b or lymphocytes and EC3d. These experiments established gp 205 as CR1.

Prior to this discovery, Dierich and Reisfeld (1975) had succeeded in solubilising C3 receptor material from a human lymphoid cell line. The protocol for this involved three main steps: (i) isolation of membrane fragments bearing receptors for C3b and C3d by nitrogen cavitation, (ii) solubilisation of the fragments in potassium bromide (2 mol $l^{-1}$) and (iii) characterisation of the receptors which revealed them to be highly complex structures. Seven years later Mussell et al (1982) carried out a more detailed characterisation of CR1 from human E. Following hypotonic lysis of E and solubilisation in potassium bromide (2 mol $l^{-1}$), the lysate obtained was passed over a C3-Sepharose column which was then eluted with potassium bromide (2 mol $l^{-1}$). Though the activity isolated in this manner could inhibit the immune adherence reaction between E and EAC14oxy23b it could not agglutinate EAC1423b and for this reason it was termed a monovalent C3b receptor. When analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) it was revealed to have a molecular weight of 55,000-66,000 daltons. This protein was thought to be the head portion of gp 205.

Fearon's (1979) method of isolating CR1 employed the use of the nonionic detergent Nonidet (NP-40). Solubilisation of E was followed by a series of chromatographic procedures, cation exchange chromatography, affinity chromatography and gel filtration, in an attempt to purify the receptor

Table 1.2 Membrane receptors for bound fragments of C3: CR1, CR2, CR3 and CR4

| Receptor Type | Specificity | Cell Type Distribution |
|---|---|---|
| CR1 | C3b, C4b, iC3b, C3i, C3c | Erythrocytes, Granulocytes, Monocytes, B and some T Lymphocytes, Kidney Podocytes Dendritic Reticulum Cells |
| CR2 | iC3b, C3dg, C3d, C3b | B Lymphocytes, Dentritic Reticulum Cells |
| CR3 | iC3b | Monocytes, Macrophages, Granulocytes, Natural Killer Cells, Dentritic Reticulum Cells |
| CR4 | iC3b, C3dg, C3d | Monocytes, Macrophages, Granulocytes |

to homogeneity. The protein isolated by this means ran as a single band on SDS polyacrylamide gels under reducing and nonreducing conditions indicating that CR1 consisted of a single polypeptide chain. In the reduced form its molecular weight was 205,000 daltons and it was a glycoprotein as indicated by its ability to bind to C3-Sepharose and lentil lectin-Sepharose. Employing a different procedure Gerdes and Stein (1980) also attempted to isolate CR1. Following solubilisation of human E in potassium bromide (2 mol $l^{-1}$), the CR1 active lysate was radioiodinated and passed over a gel filtration column. All of the material which possessed CR1 activity eluted as a single sharp peak in the void volume of the column, indicating a molecular weight of more than 100,000 daltons. Immunoprecipitation of this fraction with antisera prepared from crude CR1 active membrane fragments, revealed three bands on SDS polyacrylamide gels having molecular weights of 100,000-130,000 daltons, 80,000 daltons and 60,000 daltons. This suggested that E CR1 was a macromolecule with a molecular weight greater than 100,000 daltons and that the protein moiety consisted of non-covalently linked protein molecules of molecular weight 80,000 daltons and 60,000 daltons.

As can be seen from the preceding account the isolation and characterisation of CR1 has been a complex process. However, in the light of results from recent research workers it is now generally accepted that Fearon's view of CR1 structure is correct. Iida and Nussenzweig (1981) isolated a protein of 200,000 daltons from human E. This protein had the ability to decay dissociate both the classical and alternative C3 convertases and was also found to be a powerful inhibitor of the classical pathway C5 convertase. Using a modification of Fearon's technique Dobson, Lambris and Ross (1981) solubilised CR1 from E. Their protein had a molecular weight of 195,000 daltons in the reduced and nonreduced form and was identified as CR1 by its ability to bind to EAC3b and EAC14b in a dose dependent manner and by the blocking of rosetting activity between E and leukocytes with antiserum raised against this protein.

1.20 Polymorphism of CR1

CR1 was initially thought to be homogeneous with respect to size, but in the past few years two groups have independently described a structural polymorphism of CR1.

Previously when isolating CR1 from E it was necessary to use a large pool of donors in order to obtain a sufficient volume of blood. However by immunoprecipitation of E CR1 from individual donors Dykman et al (1983a) were able to reveal a hitherto unknown polymorphism of the receptor. Erythrocytes from each donor were radiolabelled prior to solubilisation in NP-40. The lysates were immunoprecipitated with monoclonal anti-CR1 and samples analysed by SDS-PAGE and autoradiography which revealed three types of CR1 structure, one with a major band of 190,000 daltons, the second with a major band of 220,000 daltons and the third with two major bands of 190,000 daltons and 220,000 daltons.

Characterisation of the receptors of 33 unrelated individuals revealed that the major band of 190,000 daltons, which was assigned the name "type a" was present in 70% of individuals, the major band of 220,000 daltons named "type b" was present in 3% of individuals and 27% of individuals were found to have both bands named "type c". The fact that type a CR1 structure was found in the majority of donors could be the reason why purification of CR1 from pooled donors only revealed this CR1 structure and not the less frequent types. Family studies suggested that this polymorphism was governed by two codominant alleles at a single autosomal locus. This was shown by the fact that parents who were homozygous for the type a CR1 structure only had children with type a CR1 structure. Families where one parent had type a CR1 structure and one parent had type b CR1 structure only had children with type c CR1 structure and parents who were homozygous (type a) and heterozygous (type c) had children with type a or type c CR1 structure. Thus there were postulated to be two alleles termed A for the more frequent one and B for the less frequent one.

A second group studying polymorphism of CR1 reported similar findings, (Wong, Wilson and Fearon 1983). Again two forms of CR1 were found having molecular weights of 250,000 daltons and 260,000 daltons. Individuals expressed either one of these major bands as in types a and b described by Dykman et al (1983a) or both as in type c. The higher molecular weights found by this group could be accounted for by the difference in the analytical systems used. The two bands were assigned the letters F for fast and S for slow to signify the speed at which they migrated into the gel. The F band corresponded to the 190,000 dalton band found by Dykman et al (1983a) while the S band corresponded to the 220,000 dalton band. The presence of an F band or an S band alone represented the type a or type b CR1 structure respectively while the presence of both the F and S band corresponded to the type c CR1 structure. Analyses of the occurrence of F and S forms in 15 families yielded the same conclusion as Dykman et al (1983a), viz. that these two forms were regulated by two alleles which were transmitted in an autosomal codominant manner.

Both Dykman et al (1983b) and Wong et al (1983) found that polymorphism of CR1 was also evident on peripheral blood leukocytes with these cells expressing the same phenotypes as E. Further experiments revealed the presence of an additional two alleles one coding for a CR1 of molecular weight 160,000 daltons was assigned the letter C (Dykman, Hatch and Atkinson 1984) and the other, coding for a CR1 of molecular weight 250,000 daltons was assigned the letter D (Dykman et al 1985). The 160,000 dalton band was found on individuals with either the 190,000 dalton band or the 220,000 dalton band and it was found to be most prevalent in patients with systemic lupus erythematosis (SLE). In a study of 104 unrelated normal individuals 69.2% expressed the AA phenotype, 24% expressed the AB phenotype, 3.8% expressed the BB phenotype and 2.9% the AC phenotype. No individuals expressed the CC phenotype. These phenotypic frequencies agree with the expected values

from calculations based upon the Hardy Weinberg equation for three codominant alleles at a single locus. In 45 SLE patients the frequencies for the different CR1 phenotypes were similar to normal, but three SLE patients with the 160,000 dalton band demonstrated a higher intensity of labelling in this band than normal. Since a healthy brother of one of the patients also had a large amount of the 160,000 dalton band it was considered unlikely that this was acquired and accordingly it was suggested that a heritable factor governed expression of this band. The fourth allelic form was found on one donor from a total of 200 which were tested. This donor also possessed a 190,000 dalton band and was assigned the phenotype AD (Dykman et al 1985).

Treatment of all four forms of CR1 with endoglycosidase F, an enzyme which removes both simple and complex sugars, decreased their molecular weight by 10,000 daltons while treatment with endoglycosidase H, which removes high mannose sugars, had no effect (Wong et al 1983; Atkinson and Jones 1984; Dykman et al 1985). It is therefore probable that CR1 possesses N-linked complex sugars with no high mannose sugars though it is unlikely that differences in N-linked sugars alone can account for the wide variation in the polymorphic forms of CR1. The contribution of O-linked carbohydrates is as yet unknown. These carbohydrates can account for 40,000 dalton molecular weight differences in other proteins but it remains to be determined if differences are due to this or to differences in the protein structure itself.

The functional capacities of the variants appear to be similar. Seya, Holers and Atkinson (1985) have isolated CR1 variants A, B and C and assessed their individual functional capacity as cofactors for the I mediated cleavage of C3b and for their ability to decay dissociate fluid-phase C3 convertases and found them all to be equal.

It would appear that the gene coding for polymorphism of CR1 is not linked to the human leukocyte antigen (HLA) region, which is located on chromosome six. Hatch et al (1984) reported that family studies indicated that while siblings could inherit identical CR1 phenotypes from a heterozygous parent they could inherit different HLA haplotypes from the same parent. The reverse was also true that siblings from a heterozygous parent could inherit different CR1 phenotypes and identical HLA haplotypes.

### 1.21 Biosynthesis of CR1

Little has been reported on the biosynthesis of CR1. One study on the human promyelocytic leukemia cell line HL-60 has indicated that CR1 may be initially present as a 188,000 dalton intracellular precursor (Atkinson and Jones 1984). These cells can be induced to differentiate into granulocytes by a number of components including dimethylsulphoxide (DMSO). Once differentiated these cells synthesise relatively large amounts of CR1 as assessed by their increased ability to form rosettes with EAC43b and the ability to isolate large quantities of CR1 by

immunoprecipitation with anti-CR1 antiserum or affinity chromatography on iC3-Sepharose. Following the acquisition of the receptor over various time intervals it was revealed that there was a progressive increase in CR1 over a period of two days. Pulse studies were therefore performed after two days in culture. Cells were pulsed for eight hours with $^{35}S$-methionine, or alternatively incubated for eight hours in media and then surface labelled with $^{125}I$. Comparison of cells after these different treatments revealed that while both expressed CR1 with a molecular weight of 210,000 daltons, the pulsed cells had an additional band of molecular weight 188,000 daltons. This was designated pro-CR1. Pro-CR1 could be detected at 0 hours after a 15 min pulse but it took a further 60 min for conversion to the 210,000 dalton molecular weight form to occur. The half life for the disappearance of CR1 from the cell surface was 10 hours. The molecular weight of pro-CR1 was reduced by treatment with endoglycosidase H, suggesting that it contained high mannose oligosaccharides and that it was the partially glycosylated form of CR1.

1.22 Binding Site Characteristics of CR1

CR1 is known to bind to a number of ligands apart from its major ligand C3b. These include C4b, iC3, C3c and iC3b which like C3b may all be bound in both soluble and particulate form.

Binding of solid phase C3b to human cells has been studied mainly using the rosette technique; however most of the information regarding the interaction between CR1 and C3b has come from the measurement of the direct binding of radiolabelled fluid-phase dimers and monomers of C3b to CR1. Treatment of purified native C3 with trypsin followed by application to a gel filtration column yields two distinct peaks, the first containing soluble monomeric C3b and the second dimeric C3b formed from the covalent association of C3b monomers. Arnaout et al (1981) studied the binding of radiolabelled dimeric and monomeric C3b to sheep and human E. Sheep E do not bear CR1 and did not bind significant amounts of these two ligands. Human E bound seven times more dimer and one and a half times more monomer than sheep E indicating that the dimer was more effective than the monomer in binding to CR1. Specific binding of radiolabelled monomer and dimer was followed using increasing amounts of $^{125}I$ monomer or dimer with and without increasing amounts of the appropriate unlabelled dimer or monomer. Specific saturable binding was only observed with the dimer. Scatchard plot analysis revealed that the binding affinity of the dimers to human E was $6.5 \times 10^{7}$ mol $l^{-1}$ which was similar to the binding affinity to other human cell types. Later experiments revealed that fluid-phase C3b monomer could also be bound specifically to CR1 in a saturable manner, but this only occurred in low ionic strength buffer ($\mu$=0.0513) with a binding affinity of $2.2 \times 10^{7}$ mol $l^{-1}$ which is very much lower than the affinity of the dimer under low ionic strengths ($ka=2.36 \times 10^{8}$) (Arnaout et al 1983). These data suggest that the interaction of C3b and CR1 involves ionic forces, a conclusion supported by the finding that elution of solubilised CR1 from C3-Sepharose requires the combination of high salt and nonionic detergent.

The higher affinity of the C3b dimer probably results from the interaction of the dimer with preclustered CR1 (Abrahamson and Fearon 1983). In support of this, it may be noted that oligomeric C3b prepared by cross-linking dimeric C3b with dimethylsuberimidate, has a binding affinity for CR1 which is 3.3 fold higher than C3b dimers.

As previously mentioned (1.18), iC3b is a ligand for CR1 (Ross et al 1983) although it binds at low ionic strengths and only when there is a high density of iC3b molecules ($3.5x10^4$/E) on the E.

C4b is also a ligand for CR1. Cooper (1969) was the first to show that C4b was involved in immune adherence. He found that EAC14 could adhere to E and the titre of adherence was dependent on the number of C4 molecules per E. Bokish and Sobel (1974) found that cell-bound C4b and C3b seemed to coooperate to enhance the binding of cells to EAC1423. When batches of EAC14 were prepared with differing amounts of C4, it was found that the number of C3b molecules required to achieve maximum binding was reduced if the EAC14 contained a large number of C4b molecules. In vivo this cooperative function may be of importance in the degradation of immune complexes coated with both C4b and C3b. Medof and Nussenzweig (1984) have reported that the cofactor activity of CR1 is enhanced by the deposition of clusters of C4b and C3b adjacent to one another on the substrate. They also found CR1 to be a more effective cofactor for the I mediated cleavage of C4b than C4bp when the density of C4b molecules was greater than $3x10^3$/cell. Addition of C3b to EAC14 promoted in a dose dependent fashion cleavage of C4b by I and CR1 and this enhancement was observed even when cells contained low densities of C4b molecules. The converse was also true that C4b could enhance the factor I plus CR1 mediated degradation of C3b and iC3b on EAC143b.

The CR1 binding site for C3b, iC3b and C4b is found in the c region of the C3b fragment. Ross and Polley (1975) observed that the binding of EAC143b to tonsil lymphocytes and human E could be inhibited by the addition of fluid-phase C3c. Later it was shown that fluorescent microspheres coated with C3c could bind to CR1 on E, monocytes and neutrophils and that this binding could be inhibited by anti-CR1 or fluid-phase C3b, iC3b and C3c. C3c microspheres did not bind to Raji cells which bear only CR2 (Ross et al 1983).

Whether native C3 is able to bind to CR1 without prior cleavage to C3b has occassioned considerable controversy. The issue is of importance because, since C3 is present in plasma in such high quantities, an ability to bind CR1 directly would result in the occupation of the majority of CR1 on phagocytic cells and their consequent inability to participate in multivalent interactions with C3b on opsonised particles. Berger et al (1981) showed that native C3 did not inhibit rosette formation between EAIgMC43b and PMN or E whereas C3b did. This was confirmed by Berger and Fleischer (1983) who showed the inability of C3 to block rosettes between EAIgMC43b and lymphocytes. However Sim and Sim (1981) reported that fluid-phase C3 did inhibit rosette formation between EAC143b and

tonsil lymphocytes over the same concentration range as fluid-phase C3b and that this binding was not due to cleavage of C3 to C3b. These results were similar to those of Frade and Strominger (1980), who explained the binding of C3 to lymphocytes as being due to its cleavage to C3b during the binding reaction. Dixit et al (1982) isolated CR1 from rabbit alveolar macrophages and showed that the purified receptor had a greater binding affinity for C3b than for C3. The binding affinity of C3 for CR1 could be increased by treatment with methylamine which causes hydrolysis of an internal thioester bond in C3 to reveal a sulfhydryl group which is normally only found present in the α chain of C3b which contains its labile binding site. Spontaneous hydrolysis of this bond can occur after prolonged storage or after denaturation with ammonium. This C3 is haemolytically inactive and is designated iC3. Originally, Pangburn and Müller-Eberhard (1980) reported that spontaneous hydrolysis of C3 in plasma resulted in iC3 which could bind to B and H. Dixit et al (1982) showed that this iC3 could inhibit the rebinding of purified CR1 to C3b-Sepharose as well as to C3c and C3b indicating that iC3 displays binding activity for CR1. Findings by Berger et al (1981) support the conclusion that iC3 has a binding site for CR1 since they showed that haemolytically active C3 had a much lower affinity for CR1 than did iC3. The previous reports that C3 could bind to lymphocyte CR1 may have been due to the presence of a mixture of C3 molecules with spontaneous lysed internal thiolester bonds along with haemolytically active C3. It is therefore probable that in vivo CR1 sites on phagocytic cells are unoccupied by native C3.

1.23(a) Function of Erythrocyte CR1

CR1 is the only C3 receptor which has been detected on E. Although E possess lower numbers of CR1 than any other CR1 bearing cells, the proportion of E in peripheral blood, in comparison to these other cell types, is such that 95% of CR1 in peripheral blood is found on E (Siegel, Liu and Gleicher 1981).

As already discussed, E CR1 plays a role in the prevention of lysis of bystander cells by its ability to decay dissociate convertases on these cells and by its cofactor activity for the I-mediated cleavage of C3b to the smaller degradation fragments iC3b and C3dg and the cleavage of C4b to iC4b (Iida and Nussenzweig 1981). In addition to this important function a role for E CR1 in the processing of immune complexes has been proposed. Nelson (1953) first described a role for E in immune defence after he observed that presensitised Treponema pallidium adhered to human E. Prior to this Duke and Wallace (1930) had reported the adhesion of monkey E to Trypanosomes in the presence of specific trypanosomal antibody. This clearance of microorganisms from the serum has also been observed in rabbits, guinea pigs and mice where CR1 on blood platelets can stick to bacteria and thus remove them from the circulation (Taylor et al 1985). It is now postulated that E in primates and platelets in non-primates are essential components of their respective immune systems, in that they bind opsonised soluble immune complexes via CR1 and remove

them from the circulation (Siegel et al 1981; Medof and Oger 1982; Cornacoff et al 1983; Jepsen et al 1986; Sherwood and Virella 1986). The fact that 95% of CR1 in peripheral human blood is present on E means that a circulating immune complex has a 500 to 1000 times greater chance of meeting a red blood cell than a white blood cell (Siegel et al 1981). It would be highly advantageous if red blood cells could bind to these complexes and carry them to a phagocytic cell for disposal. Such a role for E CR1 has been studied in vitro by Medof and Oger (1982) and in vivo by Cornacoff et al (1983). In vitro it was found that when radiolabelled BSA anti-BSA complexes, which had been incubated with normal serum, were added to unfractionated whole blood the majority of immune complexes became associated with the red blood cells rather than the white blood cells. The lack of immune complex binding after depletion of C3b from the serum by heat inactivation or treatment with zymosan suggested that this binding of immune complexes was to E CR1. Thus under certain conditions the E in human blood were able to compete successfully for immune complexes and remove them from the fluid-phase. The possibility that this occured in vivo was shown by Cornacoff et al (1983). Radiolabelled BSA anti-BSA complexes were infused intravenously into baboons and blood samples removed from a variety of veins after specific time intervals. The areas where E had bound immune complexes were determined by measuring the amount of radiation associated with the cells. Erythrocytes drawn from the portal vein had large amounts of associated immune complexes in comparison to E drawn from the hepatic vein. This suggested that as the blood traversed the liver, immune complexes had been removed from the E. The E themselves were returned undamaged to the circulation. The release of immune complexes from E in the liver may have resulted from the degradation of C3b in the immune complex to iC3b and C3dg by the action of CR1 and I (Medof et al 1982). Sherwood and Virella (1986) have shown that PMN and monocytes bind significant amounts of immune complexes from the surfaces of human E following incubation with immune complexes bearing E. Thus binding of immune complexes to E CR1 followed by the removal of these complexes by the phagocytic cells in the liver would not only prevent deposition of these complexes in susceptible tissues, it would also prevent overloading of CR1 bearing leukocytes in the circulation.

1.23(b) CR1 Number on Erythrocytes

The number of CR1 expressed per E appears to be highly variable among individuals. A functional variation in expression of CR1 on E from different donors was described by Miyakawa et al (1981) after the observation that the immune adherence reactivity of E could vary by as much as 10 to 100 fold in different individuals. Variations in E CR1 number have also been described quantitatively using the direct binding of anti-CR1 antibodies. This variation is termed numerical or quantitative polymorphism.

A wide variety of figures are cited in the literature to indicate the average number of CR1 molecules per E. Using a polyclonal anti-CR1

antiserum Fearon (1980) reported that there were 950 molecules of CR1 per E, while Wilson et al (1982) reported an average of 5,101 and Holme et al (1986) an average of 2,200. Using the monoclonal anti-CR1 antibody 57F, Iida, Mornaghi and Nussenzweig (1982) found an average of 1,400 CR1 sites per E and using the monoclonal anti-CR1 antibody E11, Hogg et al (1984) and Ross et al (1985) found 610 and 707 sites per E respectively.

1.23(c) Inheritance of CR1 on Erythrocytes

Wilson et al (1982) observed that the frequency distribution of E CR1 levels on normal individuals appeared to be trimodal. One group contained 34% of subjects with E CR1 numbers between 5,500 and 8,500, the second group contained 54% of subjects with E CR1 numbers between 3,000 and 5,499, and the remaining 12% of individuals had E with CR1 numbers between 1,000 to 2,999. Analysis of nine families showed that low levels of E CR1 were more frequently found in offspring of parents with low CR1 numbers and high levels of CR1 were found in offspring of parents with high CR1 numbers. They therefore proposed that numbers of CR1 on E were genetically regulated and described a simple model to explain the inheritance based on the existence of two co-dominant alleles which determine high (H) and low (L) numbers of CR1. A phenotype of HH was proposed to indicate a person who was homogeneous for the high CR1 allele which was expressed phenotypically in CR1 numbers greater than 5,500 per cell. LL was the proposed genotype for phenotypically low CR1 expression, that is, numbers less than 2,999 per E and an HL genotype was phenotypically expressed as numbers of CR1 between 3,000 and 5,500 per cell. The frequencies with which these phenotypes were observed in the normal population were consistent with the Hardy Weinberg equation for inheritance regulated by two co-dominant alleles at a single locus. Nojima et al (1985) also reported a trimodal distribution of E CR1, however others have found that E CR1 are distributed in a normal fashion (Walport et al 1985a; Ross et al 1985) or a logarithmic fashion (Holme et al 1986). Thus it may be that genetic control is more complex than originally thought involving more than two alleles.

Originally it appeared that there was no relationship between structural polymorphism of CR1 and quantitative polymorphism (Wong et al 1983). Individuals who had the AA, AB, or BB structural phenotypes were all expressed along with the HH, HL and LL numerical phenotypes. This suggested that the gene which regulated structural polymorphism was distinct from that which determined CR1 number. However a recent report by Wilson et al (1986a) described a restriction fragment length polymorphism of the CR1 structural gene involving fragments of 7.4kb and 6.9kb which appeared to correlate with variation in the numerical expression of E CR1. Four individuals with high CR1 levels had only the 7.4kb fragment, four individuals with intermediate E CR1 levels had both the 6.9kb and 7.4kb fragments and four individuals with only the 6.9kb fragment had low E CR1 expression. The presence of the restriction fragment was independent of the structural allotype expressed (Wilson et

al 1985a). These results suggested that a factor controlling quantitative polymorphism may be linked to the CR1 structural gene.

1.24 Low Erythrocyte CR1 Numbers on Patients with Systematic Lupus Erythematosus (SLE)

SLE is a disease which is associated with a number of defects in the immune system, these defects include the presence of large amounts of circulating antigen/antibody complexes (CIC) and the formation of auto-antibodies (Tan et al 1982). As previously mentioned one of the functions hypothesised for E CR1 is the processing and removal of immune complexes from the circulation. Much evidence is accumulating to suggest that the reason for the large amounts of CIC in patients with SLE is due to a defect in the ability of their E CR1 to perform this function.

While investigating the agglutination of human E by immune complexes bearing C3b it was found that the reactivity of E from patients with SLE was considerably lower than in the normal population. Out of a group of 56 SLE patients studied in Japan 37 did not show any detectable reactivity with the C3b-coated complexes, while only one out of 51 normals was found to be unreactive (Miyakawa et al 1981). This low immune adherence haemagglutination (IAHA) reactivity was shown to be a stable characteristic which persisted during active and inactive phases of the disease, suggesting that the defect was inherited and not acquired as a result of the disease state. The finding that six out of 24 healthy relatives (25%) of patients with SLE had low IAHA reactivity compared with 2% of the normal population suggested that low IAHA reactivity might be a genetic marker for SLE.

Since this initial observation additional studies have been performed which provide further support for the existence of lower CR1 numbers and impaired CR1 activity on E from patients with SLE (Iida et al 1982; Wilson et al 1982; Walport et al 1985a; Holme et al 1986). However, the main point of contention is in deciding if this abnormality is inherited or acquired. Iida et al (1982) using the monoclonal antibody 57F found that whereas the mean number of CR1 on E from 52 normal individuals was 1,410, the mean number obtained from 34 SLE patients was significantly lower at 600. Following four patients throughout phases of disease activity and inactivity revealed that while two patients expressed larger amounts of CR1 when in remission, two others did not change at all. The low level of CR1 was expressed independently of steroid therapy as treatment of asthmatics with prednisolone did not result in CR1 levels out with the normal range. In addition low levels of CR1 correlated with low levels of C4 and with high levels of CIC. These results had two possible explanations, one was that CR1 levels were inherited genetically and that the low numbers of CR1 predisposed these patients to the disease. This is consistent with the finding that CR1 levels did not change in two patients irrespective of disease activity. Alternatively the low number of receptors could have been a secondary manifestation of the disease caused by either blockade of the receptor with CIC or auto-

antibodies to CR1, or by the removal of the receptor by an unknown mechanism. This explanation is supported by the finding that two patients increased expression of CR1 numbers when in remission and by the fact that low numbers of CR1 correlated with high levels of CIC and high turnover of the complement pathway.

Wilson et al (1982) studied E CR1 from 38 SLE patients in Boston and found that the reduced numbers of CR1 could be detected on this group using both radiolabelled dimeric C3b and radiolabelled polyclonal anti-CR1. The ratio of the sites determined by the two methods was the same on both the normal and the patient group with approximately nine molecules of antibody bound per functional CR1 site. Thus the lower number of CR1 detected in the patient group was due to an absolute reduction in the number of receptors and not to a reduction in the number of functional receptor sites. Family studies showed that the healthy relatives of SLE patients expressed low CR1 numbers. The frequency distribution of patients and their relatives was very much different from the normal frequency predicted by the Hardy Weinberg equation. The families of SLE patients expressed a three- to four-fold higher frequency of the LL phenotype than did the families of normal individuals and the HH phenotype did not occur. Thus the results of Wilson and his colleagues suggested that the low CR1 numbers in SLE patients were an inherited characteristic.

Minota et al (1984) determined CR1 sites by radioimmunoassay with monoclonal anti-CR1 and CR1 activity by IAHA. The majority of SLE patients had low CR1 sites and low IAHA. The binding of radiolabelled monoclonal anti-CR1 to E and to E lysates was distributed continuously in a wide range for both normal controls and SLE patients. When however the number of CR1 sites determined on 120 controls were standardised on the basis of wheat germ agglutinin binding sites to avoid errors due to differences in E surface area, there appeared to be three distinct peaks corresponding to low, intermediate and high CR1 expression. Thirty six percent of control subjects had high CR1 sites, 53% had intermediate numbers and 11% had low numbers. Of SLE patients, the number of CR1 sites were high in 0%, medium in 52% and low in 48%. They proposed that as the prevalence of the high phenotype exceeded that predicted by the Hardy Weinberg equation, the expression of CR1 may be governed by multiple genes.

Nojima et al (1985) determined the reactivity of CR1 by measuring the capacity of E to bind to radiolabelled heat aggregated human gamma globulin (AHG) in the presence of complement. Normal E showed a range of high, intermediate and low binding capacities while E from SLE patients bound the radiolabelled complexes at a low or intermediate level but never at a high level. This low binding of SLE persisted during steroid therapy which improved other parameters of disease activity such as E sedimentation, leukocyte count and complement titre. Thus the persistence of low binding of SLE E during active and inactive phases of disease

favoured the genetic control of CR1 expression and argued against the view that CR1 expression was dependent on disease activity.

However the bulk of recent experimental evidence supports the view that reduced numbers of CR1 in patients with SLE are acquired as a result of the disease. Inada et al (1982; 1983a) reported that defective CR1 activity in SLE patients correlated with the presence of CIC in serum. This group used a new method to detect CR1 activity. Sheep E coated with antibody and C3b (EAC3b) were incubated with a detergent solubilised lysate of the test E. Depending on the amount of CR1 available in the lysate to bind with the EAC3b, when indicator human E were added there were variable amounts of inhibition of agglutination detected. Normal human E totally inhibited haemagglutination at a lysate concentration of 6% while lysate from 95% of patients with CIC present in their serum did not inhibit IAHA. When a comparison was made between patients with CIC and those without CIC, it was found that approximately 68% of patients with CIC had no inhibitory activity while only 2% of patients without IC showed no inhibitory activity. Circulating immune complexes disappeared from the patients serum as the patient entered remission and concomitant with this there was an increase in the activity of CR1. These results suggested that the defects in CR1 activity were not genetically controlled but were dependent on the availability of CR1 sites which in the active disease state may have been blocked due to the increased amount of CIC. Uko et al (1985) found CR1 deficiency in 37% of the SLE patients studied as measured by the lack of ability of these E to agglutinate in the presence of opsonised heat aggregated human IgG. Serial studies performed on E from seven normal individuals showed very little variation in IAHA titres while IAHA titres of three SLE patients varied from negative to positive IAHA and vice versa. The reduction in IAHA was usually associated with rises in serum anti-DNA binding and increases in in vivo complement activation, which are indicative of a rise in auto-antibodies and in CIC respectively. These experiments indicated that CR1 numbers were probably acquired and were in agreement with the observation of Yoshida et al (1985) who found that CR1 values on some SLE patients correlated with complement titres and changed in parallel with clinical status.

Family studies by Walport et al (1985a) have also indicated that reduced CR1 levels in SLE are acquired and not inherited. No significant difference was found between the distribution of CR1 in normal families and the distribution of CR1 in relatives of patients with SLE, findings that are in contradiction to those of Wilson et al (1982). In addition four SLE patients who were shown to express the genotype for high CR1 expression, phenotypically expressed low CR1 numbers. In agreement with these data Holme et al (1986) reported that an SLE patient with phenotypically low CR1 levels was the progeny of parents with high and intermediate genotype. These results suggest that while inheritance may play a role in determining CR1 numbers in healthy individuals, CR1 numbers in SLE patients may be modulated by certain physiological factors associated with the disease.

A major area of controversy at the present moment is whether reduced numbers of CR1 occur as a result of the actual loss of the receptor from the E membrane by proteolysis, or as a result of blockade of the receptor by CIC or auto-antibodies. Blockade of CR1 by auto-antibodies to CR1 may result in the inability to detect CR1 by polyclonal or monoclonal anti-CR1 as in the case of one SLE patient (Wilson et al 1985b). In this patient periods of disease activity were associated with absence of detectable CR1 on E and high titres of auto-anti-CR1. Uko et al (1985) have also reported a reduction in IAHA which is associated with increases in auto-antibodies to DNA. However in an earlier study Wilson et al (1982) could detect no auto-antibodies to CR1 in seven SLE patients or their relatives. In the same study blockade of CR1 by immune complexes was not considered to be a possible cause of lower CR1 numbers as only two out of 22 patients studied had serum levels of immune complexes which were out with the normal range. Ross et al (1985) found no evidence to indicate that reduced CR1 levels were due to surface-bound immune complexes as E from SLE patients did not contain abnormal amounts of C3 antigen, which is necessary for the binding of C3b or iC3b bearing complexes to CR1.

Patients with proliferative glomerular nephritis have very low or totally negative staining with anti-CR1 on frozen kidney sections. This reduction in CR1 activity is not thought to result from blockade of the receptor by immune complexes as kidney podocyte CR1 still retains the ability to bind to polyclonal anti-CR1 even after the kidney sections have been preincubated with normal human serum containing aggregated IgG, treatment which blocks the binding of podocytes to EAC3b (Kazatchkine et al 1982). This indicates that even although receptors may be blocked with complexes the antibody can still recognise and bind to an epitope on CR1. This is in agreement with the result of Hogg et al (1984) who showed that as E11 binds to a specific site in CR1 which is not part of the C3b binding site that CR1 numbers may be quantified even when receptors were occupied with soluble complexes, and with results of Ross et al (1985) who found that the binding of E11 to E CR1 was unaffected even when E were shown to be binding to circular double stranded (ds) DNA anti-dsDNA immune complexes, as assessed by their content of C3. Although most experimental data seem to imply that blockade of CR1 by immune complexes is not the reason for reduced numbers of CR1 in SLE patients, work in our own laboratory by Dr E Holme suggests that the true picture may be more complex. It was found that large opsonised immune complexes could bind to E and block uptake of both monoclonal and polyclonal anti-CR1, whereas small opsonised complexes did not block this uptake. Thus in some SLE patients reduced levels of CR1 may be due to the binding of large complexes which sterically hinder the binding of radiolabelled probes.

Alternative explanations for low CR1 numbers include the proteolytic stripping of CR1 from the E surface (Ross et al 1985; Ripoche and Sim 1986) or the presence of an inhibitory substance in SLE serum which may modulate CR1 expression. Yoshida et al (1985) suggested that CR1 levels

may be controlled by a factor in the serum of the SLE patient which causes removal of the receptor. The presence of a substance in SLE serum which may inhibit CR1 function has been suggested by the work of Ng (Personal Communication 1986). It was found that serum from an SLE patient could inhibit the binding of immune complexes to E in the presence of normal human serum. This inhibitory activity was destroyed by heat inactivation of the SLE serum. As yet there is no published work to support this theory. Experiments by Ross et al (1985) favour the view that CR1 is physically lost from the E cell surface in SLE patients. This may result from the stripping of the receptor from the membrane along with the immune complex as the E traverses the liver and spleen. His basis for this theory was the observation that SLE patients with low CR1 have a larger than normal amount of fixed C3dg molecules on their surface. Less than 100 molecules of C3dg per E were found in normal subjects while 100 to 800 molecules per E were found in patients with SLE. This larger amount of fixed C3dg was thought to have arisen from increased complement activation caused by the presence of auto-antibodies or CIC. Other diseases which were associated with complement activation such as chronic cold agglutinin disease, auto-immune haemolytic anemia Sjögrens syndrome and paroxysmal nocturnal haemoglobinuria (PNH) type 11 also had reduced E CR1 numbers and excess amounts of fixed C3dg per E. In active phases of disease SLE patients had reduced levels of E CR1 and low complement activity which correlated with high levels of fixed C3dg. As the patients entered remission E CR1 numbers increased and the levels of fixed C3dg fell. Incubation of fresh normal blood with dsDNA anti-DNA complexes in vitro, resulted in the deposition of C3dg on E, but this deposition was not accompanied by a reduction in E CR1 numbers, suggesting that neither CIC nor serum enzymes were responsible for the removal of CR1. Ross therefore explained his results by proposing that the receptor was lost during interaction of the E bearing complement coated immune complexes, with the macrophage phagocytic system during circulation through the liver or spleen. In agreement with this Walport et al (1985b) found that transfusion of E with high CR1 numbers into patients with low E CR1 numbers resulted in a gradual loss of receptors from the transfused blood. CR1 levels on the transfused blood fell from 493 to 202 CR1 sites per E over 112 hours. This was accompanied by the deposition of 410 C3dg molecules per E.

The finding of a soluble form of CR1 (Yoon & Fearon 1985) which was structurally and functionally similar to E CR1 may indicate that cellular shedding of CR1 occurs in vivo. It would be of interest to examine serum levels of CR1 in patients with SLE to determine if they are higher than normal, a finding which may lend support to the theory of increased receptor shedding in SLE.

### 1.25 Binding of DNA anti-DNA Complexes to Erythrocytes

A number of experiments have now been performed which show that reduced E CR1 numbers are associated with reduced E binding of complement coated immune complexes. Taylor et al (1983a) analysed the ability of E from

normal individuals and E from patients with SLE to bind to opsonised antibody/dsDNA immune complexes. They found that while normal individuals bound approximately 88% of a standard amount of these complexes half of the SLE patients studied had pronounced defects in their ability to bind the complexes. This defect could not be overcome by the addition of more E from the patient. This finding is consistent with the hypothesis that for immune complexes to bind to E there must be clustering of CR1 (Medof and Nussenzweig 1984). If this is the case then lower CR1 densities would result in less clustering and consequently lead to a reduced ability to bind complexes.

Horgan and Taylor (1984) studied the kinetics of binding of the DNA complexes to both normal and SLE E. Complexes opsonised in normal human serum bound rapidly within 4 minutes to normal E at 37°C and at a reduced extent at 0°C. Binding to patients E occurred very slowly requiring 30 minutes to reach equilibrium with less complexes being bound. Previously Taylor et al (1983a; b ) had reported that as less than 100 DNA anti-DNA immune complexes could bind to each normal E bearing approximately 500 CR1 sites per cell that the complexes probably bound via multivalent attachments to clusters of CR1. Clustering of CR1 requires movement of receptors in the plane of the membrane and in the case of SLE E the longer time taken to achieve maximum binding could reflect the longer time taken to organise CR1 into clusters. This theory is consistent with the finding that at temperatures below 21°C, the transition temperature for alteration of membrane fluidity, binding of complexes to normal E occurs more slowly, probably due to restriction of CR1 movement in the more rigid membrane (Zimmer and Schirmer 1974).

Thus low E CR1 in patients with SLE could contribute towards the pathogenesis of the disease by reducing the ability of the E to clear immune complexes from the circulation, thereby increasing the risk of complexes becoming deposited in the kidneys or other susceptible tissues leading to inflammation and tissue damage.

### 1.26 CR1 on Dendritic Reticular Cells

The B cell dependent areas of both the spleen and the lymph nodes contain nonphagocytic cells known as dendritic reticular cells (DRC). These cells are postulated to play a role in the retention of antigens in lymphoid follicles which is essential for the generation of B memory cells (Klaus et al 1980). It has been shown by immunofluorescence and autoradiographic studies that antigens are capable of binding to the surface of DRC and by so doing they become trapped in the follicular region in close proximity to the B cells (Nossal et al 1968). Binding of antigen/antibody complexes to DRC is mediated by complement receptors on the cells (Klaus et al 1980). This was first suggested by the work of White et al (1975) and Klaus and Humphrey (1977) in animal models. Heat aggregated human gamma globulin which had been intravenously injected into chickens was found to attach to the surface of DRC in the spleen in the absence of specific antibody, but not when the complex was treated

with pepsin such that it could not activate complement, or when the chicken was depleted of complement in vivo by treatment with cobra venom factor. This failure to bind complexes was accompanied by the failure of germinal centre formation (White et al 1975). Thymectomized mice depleted of complement by treatment with cobra venom factor were also unable to bind injected complexes to DRC which resulted in an inability to produce B memory cells (Klaus and Humphrey 1977). Gerdes and Stein (1982) were able to prove conclusively that DRC possessed complement receptors for C3 by staining frozen tissue sections with polyclonal anti-C3R. This antiserum was unable to distinguish between the various types of complement receptors, but recently using monoclonal antibodies it has been shown that DRC possess CR1, CR2 and CR3 (Hogg et al 1984; Reynes et al 1985). Monoclonal antibodies against P150/95, the proposed CR4 do not bind to DRC (Hogg et al 1986). The presence of CR1, CR2 and CR3 on these cells will thus optimise the binding of immune complexes containing C3b and its degradation fragments to the surface of the DRC.

## 1.27 Number of CR1 on PMN

Human PMN possess CR1 and CR3 and possibly also CR4. The primary function of these receptors is to establish contact between soluble immune complexes and other opsonised particles prior to their ingestion or degradation by cytotoxic reactions. PMN have been reported to possess approximately 57,000 specific binding sites for CR1 as determined by the uptake of $F(ab')_2$ anti-gp 205 (Fearon 1980). Higher numbers have been detected using the monoclonal antibody 57F,(140,000 sites/cell) (Iida et al 1982) and lower numbers are found using the monoclonal antibody E11,(46,000 molecules/cell) (Hogg et al 1984). These numbers are known to increase significantly upon exposure of cells to chemotactic stimuli such as C5a or N-formyl-methionyl-leucyl-phenylalanine (FMLP). The first indications of this were given by Anwar and Kay (1978) who reported that the eosinophil chemotactic factor of anaphylaxic (ECF-A) and histamine could selectively enhance rosettes between EC3b and human eosinophils in a dose and time dependent fashion. Human neutrophils and monocytes could also enhance complement receptors following incubation with chemotactic substances (Kay, Glass and Salter 1979). Additionally human monocytes increased complement receptor expression upon stimulation with casein, a known chemotactic agent of monocytes and after stimulation with a synthetic chemotactic peptide FMLP or after stimulation with supernatants from lymphocytes stimulated with phytohaemagglutinin (PHA) (Glass and Kay 1980). The maximum increase in complement receptors was seen by 30 minutes and this increase was dependent on the concentration of agent used and on the temperature of the incubation. At 4°C no stimulation was seen, whereas at 37°C maximum stimulation was achieved. This stimulation was specific for CR1 as there was no concomitant increase in Fc receptors. These early experiments were all performed using the rosette technique for quantification and thus it could not be distinguished between increase in CR1 number and other membrane changes which may have resulted in an increased affinity of preexisting CR1 for the ligand. Fearon and Collins (1983) provided direct evidence for the

enhancement of CR1. Employing fluorescein conjugated anti-CR1 and radiolabelled dimeric C3b they showed that C5a desarg and FMLP caused a ten-fold increase in CR1 numbers in whole blood. Whole blood was used because it was discovered that isolation procedures alone could increase the expression of CR1 on neutrophils. It was suggested that the enhancement seen with chemotactic agents was purely a result of warming the cells to 37°C and that the actual response to the chemotactic agent was minimal. Berger et al (1984) showed that FMLP did markedly enhance CR1 number over the spontaneous increase seen at 37°C. This work was confirmed by Richerson et al (1985) who reported that there was a three- to four-fold rise in CR1 following incubation at 37°C for 30 minutes and a further two- to three-fold rise upon stimulation with FMLP. Apart from chemotactic agents, phorbol myristate acetate (PMA) a tumour promoting agent which causes activation of neutrophils and monocytes (Wright and Silverstein 1982) causes an increase in CR1 number on human neutrophils. Changelian et al (1985) found that at low concentrations (4ng/ml) PMA induced an increase in CR1 expression. This increase which was initiated by PMA was abrogated by incubation with FMLP prior to incubation with PMA, indicating that the two agents draw the additional CR1 from the same source. High concentrations of PMA (greater than 16ng/ml) resulted in a decrease in CR1 expression. This decrease was not due to receptor shedding as the total cellular CR1 remained the same both before and after treatment with PMA. It was thus concluded that internalisation of CR1 had occured. The increase in receptor number which resulted from treatment with FMLP or low concentrations of PMA was not accompanied by a change in total cellular CR1 indicating that CR1 were probably being translocated from an intracellular pool. Evidence for the existence of such a pool has come from the work of O'Shea et al (1985a) who found that neutrophils which were deficient in specific granules increased CR1 expression in a normal fashion after stimulation with FMLP, although augmentation of CR3 expression was not observed. Neutrophil cytoplasts were prepared which were depleted of internal organelles and which expressed approximately 50% of the total membrane surface area of intact neutrophils. These cytoplasts were not found to increase surface expression of CR1 at 37°C or upon stimulation with FMLP suggesting that additional CR1 come from organelles within the cell. To determine if this might be the case, normal neutrophils were fixed with acetone to allow fluoresceinated monoclonal anti-CR1 to penetrate the membrane and bind to internal proteins. After this treatment a large amount of internal fluorescence was seen. An attempt was made to locate the organelle which contained the intracellular pool of CR1. Neutrophils were disrupted and fractionated on sucrose gradients to obtain plasma membrane, specific granule, and azurophilic granule enriched fractions. Radiolabelled monoclonal anti-CR1 bound to the plasma membrane enriched fraction but not to any of the other fractions. It was suggested that CR1 could possibly be located in the Golgi apparatus as this organelle was present in the plasma membrane enriched fragment. CR3 was thought

to be located in the specific granules in agreement with the work of Todd et al (1984).

Evidence for the in vivo increase in CR1 expression on neutrophils has come from studies on patients undergoing haemodialysis. This treatment results in activation of the complement system due to exposure of blood to fresh cellulose membrane which activates the alternative pathway. C5a which is generated by this activation may be the in vivo stimulus which augments CR1 expression on neutrophils. In vitro a direct relationship was shown between CR1 numbers on neutrophils and the concentration of C5a added. The ability to augment expression of CR1 on neutrophils may be an important mechanism for increasing phagocytic activity at sites of inflammation in vivo (Lee, Hakim and Fearon 1984).

### 1.28 Function of CR1 on Phagocytic Cells

The function of CR1 on phagocytic cells is primarily concerned with the binding of cells to particles opsonised with fragments of C3 degradation which are ligands for the receptor, namely C3b, iC3b, C4b, C3c and iC3. Attachment of CR1 to opsonised particles does not necessarily lead to their ingestion. Originally it was proposed that CR1 acted in synergy with the Fc receptor on phagocytic cells to promote ingestion with CR1 acting exclusively to establish contact between the cell and the opsonised particle and ligation of the Fc receptor being required to directly trigger ingestion. Ehlenberger and Nussenzweig (1977) found that the presence of C3b on a particle could reduce by 100-fold the amount of IgG required to produce ingestion but other non-immunological agents which brought phagocyte and particle into close contact could also reduce the amount of IgG required for ingestion. It was thus concluded that while CR1 may be necessary to establish a strong contact it had no direct role in the phagocytic process. Newman and Johnston (1979) provided evidence to support this conclusion, when it was observed that sheep E coated with C3b were bound but not ingested by neutrophils. Addition of small amounts of IgG to the sheep cells triggered ingestion and release of superoxide anions .

Studies of the CR1 receptor on resident and thioglycollate elicited mouse peritoneal macrophages revealed that nonactivated macrophages bound to sheep EAIgMC3 but did not ingest them to a significant degree, while activated macrophages bound and ingested these complexes even when Fc receptors were blocked by an anti-macrophage IgG fraction (Bianco, Griffin and Silverstein 1975). As thioglycollate elicited macrophages behave similarly to macrophages that have been stimulated with lymphokine Griffin and Griffin (1979) examined the effect on macrophages of a soluble factor produced from T cells. They found that macrophages treated with this factor could be converted in vitro from only mediating binding to EAIgMC3b to promoting ingestion of this complex. Thus complement receptors on mouse peritoneal macrophages could exist in two states an inactive one which mediated only binding and an activated one which mediated both binding and ingestion.

In an attempt to determine the mechanism whereby this transition took place, Griffin and Mullinax (1981) studied the topography of CR1 on active and inactive mouse peritoneal macrophages. Previously it had shown that macrophages plated on coverslips coated with IgG containing immune complexes or opsonised IgG immune complexes could mobilise their Fc and complement receptors in the plane of the membrane so that they accumulated in the basal portion of the membrane in contact with the substrate leaving the surface void of these receptors (Michl et al 1979). When inactive macrophages were plated onto surfaces bearing unopsonised IgG containing immune complexes they could bind to but not ingest sheep EAIgMC3b. Macrophages treated with lymphokine could ingest sheep EAIgMC3b while plated onto these same surfaces, but binding and ingestion was abolished by plating the activated macrophages onto surfaces coated with opsonised IgG containing immune complexes. Binding of EAIgMC3b by nonactivated macrophages was not altered on surfaces containing opsonised IgG containing immune complexes. These results were explained by proposing that treatment with the lymphokine resulted in the ability of CR1 to trigger ingestion and also to become mobile in the plane of the membrane. Inactive macrophages could not mobilise CR1 in the plane of the membrane as indicated by the retention of rosetting ability with EAIgMC3b while plated on complement coated surfaces thus it was postulated that activation of CR1 resulted from its release from a normally anchored position allowing it to become active in phagocytosis.

PMA was found to be capable of promoting ingestion of sheep EAIgMC3b in cultured human monocytes. Wright & Silverstein (1982) plated monocytes, which had been pretreated with PMA, onto IgG coated surfaces. These cells were able to both bind and ingest sheep EAIgMC3b and iC3b whereas control monocytes could only bind these complexes. However control monocytes were able to modulate CR1 in the plane of the membrane unlike unstimulated mouse macrophages, which indicated that mobility alone was insufficient to promote ingestion. To date the biochemical reaction which accounts for the transition of CR1 from an inactive state to an active one remains unknown although recent evidence suggests that phosphorylation of the receptor may occur. Changelian and Fearon (1985) studied phosphorylation of CR1, CR2 and CR3 in phagocytic and nonphagocytic cells. Neutrophils, monocytes and lymphocytes were labelled with $^{32}PO_4$ and incubated in buffer alone or in buffer containing PMA which is known to increase protein phosphorylation. After an appropriate time interval CR1 was immunoprecipitated from cell lysates and analysed by autoradiography. CR1 from unstimulated cells was not phosphorylated whatever the length of incubation time. PMA stimulation induced phosphorylation of CR1 in monocytes and neutrophils and CR2 in B lymphocytes. CR3 was not phosphorylated in any of the cells and neither was the Fc receptor. Phosphorylation seemed to correlate with acquisition of a phagocytic function in monocytes and neutrophils. CR1 from unstimulated monocytes did not promote ingestion of EAIgMC3b nor did it become phosphorylated whereas upon stimulation with PMA CR1 was phosphorylated and ingestion occurred. Acquisition of a phagocytic function by neutrophils requires prior stimulation with chemotactic

factors before activation of CR1 by PMA occurs. Treatment of neutrophils with FMLP and PMA resulted in phosphorylation of CR1 with the concomitant ability to ingest EAIgMC3b. These authors thus suggested that phosphorylation of CR1 may be the structural basis for the activated state of CR1.

In addition to PMA other proteins have been found which may represent physiological regulators of complement receptor function in vivo. Pommier et al (1983) found that soluble fibronectin (fn) could induce peripheral blood monocytes to ingest sheep EAIgMC3b and could also increase the phagocytosis of EAIgG. This activation was not as a result of denovo synthesis of CR1 as the response was initiated very quickly and could not be prevented by protein synthesis inhibitors. It could however be inhibited by the addition of colchicine a microtubule depolymeriser. Wright, Craigmyle and Silverstein (1983a) found that substrate-bound fn and substrate-bound serum amyloid P component caused activation of both CR1 and CR3 on cultured human monocytes. These monocytes bound to fn-coated surfaces by way of a fn receptor. Ligation of this receptor on the basal surface of the membrane resulted in activation of complement receptors over the whole cell membrane. Collagen and fibrin can also bind to fn receptors, these are proteins which are usually present at sites of tissue injury and could be important in vivo for activation of complement receptors in areas of inflammation.

Neutrophils were not initially found to be activated by fn (Wright et al 1983a). However when they were exposed to chemotactic factors such as FMLP, fn was found to activate phagocytosis of EAIgMC3b (Pommier et al 1984). This could be an important in vivo mechanism for effectively limiting neutrophil phagocytosis to sites of inflammation where they have accumulated as a result of chemotaxis. Both monocytes and neutrophils bear a receptor for C1q through which they can attach to C1q-coated immune complexes. This binding is inefficient and can be enhanced by preincubating the cells with fn, which leads to ingestion if C3b or iC3b are present on the complex. Fibronectin may act by enhancing the ability of C1q receptors to bind to C1q or alternatively fn may itself bind to C1q to promote a stronger interaction between immune complex and phagocyte. Whatever the explanation the presence of C1q, C3b or iC3b on immune complexes greatly enhances their phagocytosis by fn-stimulated monocytes. (Bohnsack et al 1985, Sorvillo, Gigli and Pearlstein 1986).

CR1 undoubtedly plays an important role in phagocytosis by its ability to promote strong binding between phagocytic cells and opsonised particles; however CR3 may be much more effective in triggering ingestion and mounting a respiratory burst than is CR1. Wright and Silverstein (1982) reported that PMA treated monocytes which were bound to surfaces coated with IgG and C3b were still able to bind and ingest EAIgMC3bi by way of CR3. The reverse was also true that monocytes bound to surfaces coated with iC3b and IgG could bind and ingest EAIgMC3b by way of CR1 however CR3 was ten times more effective than CR1 in ingestion of these

complexes. Ross, Cain and Lachmann (1985) observed that rabbit E coated with C3b (RaEC3b) were ingested by monocytes and neutrophils. Addition of monoclonal anti-CR3 blocked the ingestion of these E complexes but did not affect the binding of the phagocytic cell to E. This implies that the CR1 mediated binding of RaEC3b to phagocytes does not induce ingestion, but that a synergism between CR1 and CR3 may exist where CR1 promotes strong binding which allows weak binding to CR3 which in turn triggers ingestion.

1.29 Endocytosis by PMN

Phagocytosis of large particles and pinocytosis of soluble material and small particles are two processes collectively termed endocytosis. CR1 of unstimulated neutrophils while being unable to initiate phagocytosis can pinocytose small particles and soluble complexes into clathrin-coated pits. Fearon, Kaneko and Thomson (1981) noted that PMN preincubated with $F(ab')_2$ anti-CR1 fragments at 0°C and then subsequently treated with a fluorescent second antibody, tetramethylrhodamine isothiocyanate (TRITC)IgG $F(ab')_2$ fragments demonstrated a patchy fluorescence while PMN treated in the same way at 37°C showed very few fluorescent clusters and in some cases none at all. Thus at 37°C anti-CR1 $F(ab')_2$ fragments were not accessible to the fluorescein labelled second antibody as they had been internalised. Subsequent experiments revealed that more than 90% of the bivalent $F(ab')_2$ anti-CR1 which bound at 37°C became internalised, while monovalent anti-CR1 Fab fragments were not internalised. Thus cross-linking of the receptors was required for internalisation to proceed, an indication that the trigger for endocytosis required the binding of the ligand to several CR1 simultaneously.

Incubation of PMN with cytochalasin B, an agent which disrupts microfilaments (MF) did not affect the uptake of $F(ab')_2$ anti-CR1 implying that the structural mechanisms leading to this type of ingestion differed from that involved in phagocytosis a process which is inhibited by cytochalasin B (Allison, Davies and De Petris 1971). It was therefore proposed that internalisation was mediated by a structural protein termed clathrin. Many nonphagocytic cells have the ability to internalise membrane receptors and their ligands as a means of taking up nutritional and regulatory proteins from the extracellular fluid. This is achieved by the clustering of receptors in specific regions of the plasma membrane which overlie clathrin-coated pits. These invaginate into the cell to form coated vesicles which transport the receptor-ligand complex to lysosomes where the internal contents are degraded (Goldstein, Anderson and Brown 1979). Abrahamson and Fearon (1983) were able to visualise the intracellular distribution and fate of bound anti-CR1 $F(ab')_2$ fragments on PMN and monocytes, using a fluorescent- or ferritin-conjugated second antibody. In both cell types upon warming to 37°C for 20 minutes bright fluorescence was observed in intracellular bodies. The ferritin label showed that at 37°C the complexes were taken into clathrin-coated pits within five minutes and subsequently delivered to azurophil granules in PMN and lysosomes in monocytes. Hogg et al (1984)

reported that the monoclonal anti-CR1 antibody, E11, was not endocytosed by PMN unless it was firstly cross-linked with $F(ab')_2$ anti-mouse Ig. Changelian et al (1985) studied the uptake of the monoclonal anti-CR1 monoclonal antibody, YZ-1, by neutrophils in response to PMA. High concentrations of PMA (over 16ng/ml) resulted in internalisation of the receptors. Internalisation occurred when monovalent antibody fragments were bound to CR1 indicating that PMA allows endocytosis to proceed even in the absence of a cross-linked ligand. O'Shea et al (1985b) also found that a second phorbol ester, phorbol dibutyrate (PDBu) caused internalisation of anti-CR1 Fab fragments by neutrophils which had been stimulated with FMLP and also by unstimulated neutrophils. Monocytes and macrophages spontaneously internalised Fab fragments, but this could be considerably enhanced by addition of PDBu or PMA. Endocytosis of soluble complexes and small particulate material which can be contained within the 100nm clathrin-coated pits may provide an important additional clearing mechanism at sites of tissue injury.

## 1.30 Role of the Cytoskeleton in Endocytosis

In an attempt to understand the mechanisms whereby ligation of CR1 causes endocytosis the distribution and lateral mobility of CR1 in the plane of the neutrophil and monocyte plasma membrane has been studied. Clustering of receptors is a prerequisite for the endocytosis of many ligands. Some receptors require first to bind to their ligand before accumulating in regions of the plasma membrane which overlie clathrin-coated pits, others reach these areas without any involvement of their ligand. In the former case ligation of receptors may result in an alteration in the interaction with the underlying cytoskeleton and in the latter case receptors may be actively moved or passively moved as a result of membrane flow. Petty et al (1980) reported that CR1 appeared to be present in discrete clusters on the membranes of PMN and monocytes. This clustered distribution was observed with both bivalent and monovalent FITC labelled anti-CR1 Fab fragments which indicated that the clustering was not due to cross-linking by the antibody. No redistribution of fluorescence was seen after photobleaching indicating that CR1 was not free to move in the plane of the membrane. Hafeman et al (1982) subsequently showed that plating neutrophils onto glass slides, as had been the procedure in the previously described experiment, caused the redistribution into clusters. Plating onto slides coated with a lipid monolayer to which neutrophils do not adhere allowed CR1 to maintain their initial uniform distribution. Neutrophils which adhered to glass coverslips displayed an erratic fluorescence recovery when measured using the photobleaching technique indicating that the clustered receptors were not freely diffusing whereas those plated onto a lipid monolayer showed a recovery consistent with free mobility in the plane of the membrane. These findings may indicate that CR1 is not preclustered but is normally uniformly distributed throughout the membrane and only upon binding to a particular surface or to its ligand does it become redistributed into clusters. This redistribution may involve an association with the cytoskeleton an intricate network of microfilaments (MF), 10nm filaments

and microtubules (MT) which lies just underneath the cell membrane. It is thought that MF may attach to specific integral membrane proteins and cause them to be actively moved in the plane of the membrane. This kind of movement is energy dependent and sensitive to calcium concentration.

In an attempt to follow the involvement of the cytoskeleton in the redistribution of CR1 and Fc receptors on neutrophils Jack and Fearon (1984) employed two drugs which interfere with MF function; chloropromazine which alters calcium concentration and cytochalasin D which is one of a class of compounds isolated from the culture fluid of certain moulds, which can block the polymerization of MF. Neutrophils labelled with $F(ab')_2$ anti-CR1 followed by a TRITC conjugated second layer antibody showed a patchy fluorescence after incubation for five minutes at 30°C. Capping of unoccupied CR1 sites was also observed and all caps were associated with an intense staining of myosin underlying the area of the cap. Previously Jack and Fearon (1983) had reported that neutrophils labelled with $^{125}I$-$F(ab')_2$ anti-CR1 and then solubilised had 83% of this ligand associated with the insoluble cytoskeleton, while only 5% of a monovalent ligand remained in association with the cytoskeleton thus indicating that cross-linking of CR1 resulted in an association with the cytoskeleton. Chloropromazine and cytochalasin D were both found to inhibit the redistribution of cross-linked CR1 into patches providing further support for the involvement of MF in the redistribution of CR1. An additional finding was that capping of CR1 was accompanied by the co-capping of Fc receptors while distribution of other receptors was not altered. The reverse was also true that capping of Fc receptors resulted in co-capping of CR1. Both types of receptors become located in the same areas of intense myosin staining. This has led to the hypothesis that both types of receptor interact with the cytoskeleton such that redistribution of one causes the same altered distribution of the other. This might explain why phagocytosis is synergistically enhanced following the opsonisation of an IgG coated particle with C3b (Ehlenberger and Nussenzweig 1977).

Internalisation of monovalent anti-CR1 Fab fragments by neutrophils and monocytes in response to PMA and PDBu is also considered to arise as a result of association of CR1 with the cytoskeleton. O'Shea et al (1985b) showed that treatment of these cells with cytochalasin B prior to stimulation with phorbol esters abrogated internalisation of Fab anti-CR1 fragments. This group also found that PDBu induced a time dependent association of CR1 with the cytoskeleton and they suggested that phorbol ester mediated internalisation is as a result of association of CR1 with the cytoskeleton.

The exact role of MT in endocytosis is not clear although they are thought to be responsible for the movement of other surface proteins away from the area of plasma membrane where specific receptors are being internalised. Colchicine, an alkaloid derived from the autumn crocus Colchicum autumnale is a potent inhibitor of many cell functions which involve MT. As colchicine prevents the movement of proteins which are

not involved in the internalisation process, it is probable that MT are involved in the localisation of membrane proteins. In support of this theory, capping of Concanavalin A receptors is induced by colchicine suggesting that these receptors are held in place by MT and only upon depolymerization of MT do the receptors become free to move in the plane of the membrane.

1.31 CR1 on B Lymphocytes

Lay and Nussenzweig (1968) were the first to describe a complement receptor on blood lymphocytes after they observed that lymphocytes would not form rosettes with EAIgM unless the EAIgM had first been incubated with serum. The receptor was later shown to be specific for both C3b and C4b (Ross and Polley 1975). It is now known that the majority of all B lymphocytes from the spleen and peripheral blood express CR1 (Tedder et al 1983) while 75%-85% of peripheral blood B lymphocytes also express CR2 (Ross et al 1978). B lymphocytes are not thought to express CR3 (Ault and Springer 1981).

CR1 levels on B lymphocytes have been quantified by the use of polyclonal and monoclonal anti-CR1 antibodies and by a C3b dimer. The numbers reported by these various means are 20,000 (Fearon 1980), 360,000 (Iida et al 1982) and 21,000 (Arnaout et al 1981). Fearon and Collins (1983) found that unlike PMN these numbers do not spontaneously increase upon warming to 37°C nor upon stimulation with chemotactic factors. They therefore suggested that B lymphocytes express all their CR1 on the plasma membrane and do not have any intracellular pools of CR1 available for translocation. However Sim and Sim (1983) found that the rate of cleavage of C3b to iC3b in the presence of I and B lymphocytes (a CR1 source) was greatly increased if the cells were rendered permeable by the addition of detergent indicating that B lymphocytes may have intracellular pools of CR1.

The ontogeny of CR1 on B lymphocytes was initially studied by Tedder et al (1983). Using the technique of indirect immunofluorescence with $F(ab')_2$ anti-CR1, it was shown that B lymphocytes gradually acquire the ability to express CR1 as they become more mature. Fifteen percent of large pre-B cells, 35-48% of small pre-B cells, 60-80% of immature B cells and 99% of mature B cells were found to express CR1. The receptor was lost when B cells differentiated into mature plasma cells.

The function of CR1 on B cells is still largely speculative. Iida and Nussenzweig (1983) showed that CR1 on B lymphocytes could inhibit the formation of C3 and C5 convertases on sheep E. This inhibition of convertase formation could be reversed by approximately 60% by the addition of fluid-phase C3b or monoclonal anti-CR1. In the presence of I, lymphocyte CR1 could also mediate the release of a C3c fragment from EAC14oxy23b. Thus CR1 on lymphocytes, possibly in combination with membrane cofactor protein, may act to protect cells from damaging effects

of complement activation caused by immune complexes trapped in areas of B cell activation such as the lymphoid tissue.

B lymphocyte CR1 is also thought to play a role in the triggering of an antigen specific response by binding to immune complexes containing C3 and activating B cell proliferation. In vitro studies performed by Daha, Bloem and Ballieux (1984) have shown that high doses of $F(ab')_2$ anti-CR1 can enhance in a dose-dependent fashion the production of IgG by B lymphocytes, when these cells in the presence of T cells and monocytes have been stimulated with sub-mitogenic doses of pokeweed mitogen (PWM). This suggests that if ligation of more than one CR1 on B lymphocytes occurs it can lead to the modulation of antibody production, although ligation of CR1 on T cells and monocytes, the other cell types necessary for a response to PWM, cannot be ruled out as a prerequisite for the B lymphocyte response. Experiments performed by Frade et al (1985b) have indicated that CR2 may also be involved in the proliferation of B lymphocytes. Thus CR1 and CR2 may cooperate in the activation of B cells.

1.32 CR1 on Podocytes

The existence of a complement receptor on the human renal glomerulus was first suggested by Gelfand, Frank and Green (1975) after the observation that sheep E or bacteria coated with C3b could adhere to the glomeruli in frozen sections of human kidney. This finding was supported by the work of Carlo, Nagle and Shin (1978) who observed that C3b coated fluorescein labelled bacteria could bind to human renal tissue. The receptor was thought to be CR1 as treatment of C3b with H and I resulted in the generation of iC3b which did not bind to the kidney. A year later sheep E coated with iC3b were shown to bind to the glomeruli and it was thus concluded that perhaps both CR1 and CR3 were present in this tissue (Carlo et al 1979). Since that time iC3b has been shown to be a ligand for CR1 and monoclonal antibodies against CR2 and CR3 have not been found to bind to kidney sections (Kazatchkine and Fischer 1984). In addition sheep E coated with C3dg and C3d do not adhere to kidney sections indicating that only CR1 is present in the glomerulus (Fischer et al 1986).

CR1 is located exclusively in the podocytes of the glomerulus (Kazatchkine et al 1982; Emancipator et al 1983), and Fischer et al (1986) have estimated that there are approximately 200,000 CR1 molecules per podocyte. CR1 is distributed homogeneously on the plasma membrane of these cells and as CR1 is seen in the Golgi apparatus it is probable that podocytes synthesise the receptor. There are two main proposed functions for CR1 on kidney podocytes. The first is a protective function in that it may behave similarly to CR1 on other cell types in being able to decay dissociate C3 and C5 convertases and being able to act as a cofactor for the I mediated cleavage of C3b (Iida and Nussenzweig 1983; Fischer et al 1986). In this way CR1 on kidney podocytes may help prevent complement activation on the basement membrane of the glomerulus. Secondly, CR1 may be able to directly mediate adsorptive endocytosis of soluble complexes

which bear C3b. These complexes may filter through the basement membrane if it has been altered in some manner by a disease process (Fischer, Appay and Kazatchkine 1984).

Altered patterns of staining on podocytes are seen in patients with proliferative SLE nephritis (Kazatchkine et al 1982). These patients have very low or totally negative staining with anti-CR1 whereas patients with non proliferative nephritis have normal staining. SLE is a disease which is associated with the deposition of antigen/antibody complexes in the glomeruli therefore low numbers of CR1 on the kidney podocytes of these patients with proliferative nephritis may affect the efficiency of immune complex processing.

1.33(a) CR2 Structure

Barel, Charriaut and Frade (1981) isolated a CR1 like molecule from the membranes of Raji cells. This protein had a molecular weight of 140,00 daltons and it bound to both C3b-Sepharose and lentil lectin-Sepharose. For this reason the protein termed "gp 140" was assumed to be a receptor similar to CR1. However in 1983 and 1984 two groups presented evidence to suggest that gp 140 was in fact the C3d receptor CR2. Iida, Nadler and Nussenzweig (1983) investigated the possibility that a surface marker called, "B2" purified from human B lymphocytes, and CR2 were identical. Gp 140 was isolated from Raji cells and tonsil lymphocytes. This protein had the same molecular weight as the B2 marker and it was found to have an affinity for monoclonal antibodies to B2 and also to C3 fragments. Anti-B2 partially inhibited rosette formation between tonsil lymphocytes and EAC3d. Total inhibition was only observed if the lymphocytes were first incubated with anti-mouse IgM, thus indicating that inhibition of rosette formation was as a result of cross-linking of B2 receptors and not a direct competition between B2 and C3d. These experiments suggested that the 140,000 dalton B2 antigen was CR2.

A second lymphocyte surface marker postulated to be CR2 was a protein of molecular weight 145,000 daltons recognised by the monoclonal antibody HB5 (Weis, Tedder and Fearon 1984). HB5 induced partial inhibition of rosetting between EC3d and Raji cells. Again total inhibition was only achieved by first cross-linking the HB5 receptors with mouse IgG. When detergent lysates of Raji cells were passed over a protein A column to which HB5 was bound, a 145,000 dalton protein was extracted from the lysates and conferred on the protein A particles the ability to bind specifically to EC3d or EC3bi. This directly demonstrated the CR2 function of HB5. Neither monoclonal antibodies, anti-B2 or HB5 mask the ligand binding site of CR2 as they do not prevent the binding of C3d to CR2 without prior cross-linking of CR2 by a second antibody. For this reason it was uncertain if they did actually represent CR2. A polyclonal antisera raised against gp 140 did prevent rosetting of EC3, EC3b and EC3d with Raji cells without a second antibody being necessary.

A series of experiments by Frade et al (1985a) confirmed the identity of gp 140 as the C3d/C3dg CR2 and also showed that CR2 was recognised by anti-B2, HB5 and OKB7. The epitopes of CR2 recognised by these three antibodies are distinct as binding of one antibody does not prevent the uptake of the others.

Previous to these studies a 72,000 dalton glycoprotein had been isolated from the medium of B lymphoblastoid cells (Lambris, Dobson and Ross 1981). Antibodies prepared against this protein could inhibit lymphocyte rosette formation between EAC3d but not EAC3b. Gp 72 was also shown to bind to EAC3d but not EAC3bi and it was this concluded that B type lymphoblastoid cells synthesis and secrete a C3d binding protein into their surrounding culture medium and that this protein resembles CR2. Recently it has been reported that gp 72 can bind to soluble $^{125}$I-C3b and induce a cleavage in the C3b molecule (Conseiller et al 1985). It was proposed that gp 72 was a serine protease which may function to augment cleavage of C3b as well as perhaps representing the fragment of gp 140 which contains the C3d binding site.

1.33(b) Binding Site Properties of CR2

Binding sites for CR2 were first shown to be present in both C3d and iC3b (Ross et al 1973b; Ross and Lambris 1982). Ross et al (1983) showed by the binding of fluorescent microspheres coated with C3dg to monocytes and Raji cells that CR2 also had a binding site for C3dg. It was controversial however if C3b bound to CR2 or not. Frade et al (1985a) showed that Raji cells which do not possess CR1 (Lambris, Dobson and Ross 1980; Iida et al 1982; Tedder et al 1983) had the ability to bind C3b and that this activity was as a result of binding to CR2. All Raji cell rosettes between EAC3b, EAC3bi, EAC3dg and EAC3d were completely inhibited by anti-gp 140. Rosettes formed between Raji cells and EAC3b required approximately 100-fold more fixed C3b molecules per E than fixed EAC3bi, C3dg or C3d. This indicated that CR2 probably has a low affinity for the C3d region of uncleaved C3b.

B lymphocytes bear a membrane receptor specific for the Epstein Barr virus (EBV) (Jondal and Klein 1973). EBV is a ubiquitous virus which infects essentially all the normal adult population. In vivo it is associated with malignancy of many epithelial cells and in vitro it induces B cell proliferation with the concomitant ability to secrete immunoglobulins. When the EBV receptor was first discovered its biochemical nature was unknown but since that time evidence is accumulating to suggest that it is identical with CR2. Yefenof et al (1976) performed studies with two colour staining in order to visualise the relationship between the two receptors on human B cells. They found that two colour fluorescence staining of EBV receptors and CR2 showed complete overlapping of red and green fluorescence. In addition capping of EBV receptors induced co-capping of CR2 suggesting a close association between EBV receptor and CR2 on B cells. Hutt-Fletcher et al (1983) isolated fluid-phase CR2 which bound to C3d fragments on E and which

could inhibit rosettes between EC3d and B cells. It could not however inhibit the binding of B cells to the EBV. Isolated EBV receptor was found to inhibit EBV binding to B cells, but did not inhibit binding of EC3d to B cells. It was therefore concluded that CR2 was probably not the binding site for EBV. This group had however only isolated gp 72 which is now known not to be the intact CR2. Fingeroth et al (1984) found that the rank order of binding of fluorescent labelled EBV to four lymphoblastoid cell lines was identical to the order of binding of HB5 and anti-B2, also binding to EBV to a B lymphoid cell line could be inhibited by pretreating the cells with HB5 and a second antibody to cross-link the receptors. Finally when CR2 was linked to Staphylcoccus aureus (S. aureus) by means of HB5, it resulted in the ability of the bacteria to bind $^{125}$I-labelled EBV. Nemerow, Siaw and Cooper (1985) isolated gp 145 from Raji cells. A dot-blot immunoassay demonstrated the ability of gp 145 to bind to either EBV or C3dg indicating that purified gp 145 had a binding site for both C3d and EBV. Mold, Cooper and Nemerow (1985) incorporated purified CR2 into phospholipid liposomes which were then found to bind to EC3d. This binding was inhibited by OKB7. CR2 liposomes bound to a cell line which expressed the EBV membrane antigen but could not bind if these cells were treated with a virus inhibitor. Thus to date the existing experimental data suggests that CR2 and the EBV receptor are identical.

1.33(c) Function of CR2

CR2 is expressed exclusively on B cells (Ross 1980) and as such it probably plays some role in the regulation of B cell function. Frade et al (1985b) have provided some evidence that CR2 is involved in the B cell proliferative response to T cell products. ACA-54 a B cell growth factor (BCGF) produced from activated T cells caused proliferation of B cells. This effect was potentiated by the addition of anti-$\mu$-antibody which is another stimulator. F(ab')$_2$ fragments of anti-gp 140 were prepared and these enhanced in a dose dependent manner the proliferation of B cells in the presence of ACA-54, but not in its absence. The mechanism whereby CR2 exerts this effect is unknown.

Changelian and Fearon (1985) have shown that PMA induces phosphorylation of CR2 in B cells, while no phosphorylation of CR1 occurs. This may indicate that CR2 rather than CR1 may be more important in regulation of B cell function.

1.34(a) CR3 Structure

The structure of CR3 like CR2 has been elucidated with the aid of monoclonal antibodies. The rat anti-mouse monoclonal antibody anti-Mac1 which defines the antigen M170 or Mac1 on the surface of mouse macrophages has been of particular importance in this respect (Ho and Springer 1982). M170 is expressed on mouse thioglycolate elicited macrophages and on other mouse macrophages, blood monocytes, granulocytes and NK cells but is absent from lymphocytes. Anti-Mac1

cross reacts with the same cell population in humans (Ault and Springer 1981) and it is probably identical to the OKM1 antigen (Breard et al 1980) and the Mo1 antigen (Todd et al 1982).

The function of this antigen when it was first discovered was unknown although it had been structurally characterised and found to contain two noncovalently associated α and β subunits with molecular weights of 170,000 daltons and 95,000 daltons respectively (Kurzinger and Springer 1982). Beller et al (1982) presented evidence that this antigen was intimately associated with or actually represented CR3. They found that mouse macrophages were inhibited from forming rosettes with EAC3bi by prior incubation of the macrophages with anti-Mac1. In confirmation of this hypothesis Wright et al (1983b) showed that EAC3bi could bind to S aureus particles coated with OKM1 antigen in a similar fashion to their ability to bind to phagocytic cells bearing CR3.

Interest in the Mac1 antigen has been further aroused by the finding that it is structurally related to an antigen on cytotoxic T cells. These T cells can recognise the presence of a foreign antigen on the surface of a particular target cell when it is in association with the correct major histocompatability (MHC) products (Springer et al 1982). Monoclonal antibodies have been used as probes to determine the surface component on the lymphocyte membrane which is responsible for recognising and binding to target cells. A monoclonal antibody against the lymphocyte function associated antigen (LFA-1) was consistently found to inhibit killing by cytotoxic T lymphocytes. Thus LFA-1 was postulated to participate in the magnesium-dependent antigen recognition and adhesion step of cytotoxic T cell-mediated killing. LFA-1 has also been shown to be present on B cells, granulocytes and monocytes and to be involved in natural killing and T helper cell responses.

LFA-1 contains an α chain of molecular weight 180,000 daltons and a β chain of molecular weight 95,000 daltons (Kurzinger and Springer 1982). The β chains of both Mac1 and LFA-1 are highly homologous although the α chains are found by tyrosyl tryptic peptide mapping to be quite different. Monoclonal antibodies against Mac1 and LFA-1 do not cross react as revealed by immunoprecipitation experiments. Anti-Mac1 immunoprecipitates Mac1 from macrophages whereas LFA-1 can not. Anti-LFA-1 immunoprecipitates LFA-1 from con A blasts but anti-Mac1 does not. In contrast to this polyclonal antisera prepared against purified Mac1 can immunoprecipitate both Mac1 and LFA-1. This suggests that the cross reaction is between the β subunits and not the α subunits.

Ho and Springer (1983) studied the biosynthesis and assembly of the α and β subunits of Mac1. Their results are consistent with the hypothesis that the two subunits of Mac1 are synthesised separately from different messenger RNA's and then they are associated noncovalently to form α,β complexes.

A third protein with structural similarities to Mac1 and LFA-1 was described by Sanchez-Madrid et al (1983). Monoclonal antibodies against the 95,000 dalton β subunit immunoprecipitated three α chain structures from $^{125}$I-labelled granulocytes. These were the α subunit of LFA-1 (180,000 daltons), the α chain of Mac1, (170,00 daltons) and an additional subunit of 150,000 daltons. This new α subunit was found to be covalently associated with a β subunit of 95,000 daltons in an $\alpha_1\beta_1$ structure similar to Mac1 and LFA-1. This new glycoprotein termed P150/95 was a distinct protein and not a degradation product or an immature form of Mac1 or LFA-1. Thus Mac1, LFA-1 and P150/95 appear to be a family of related polypeptides with a common β subunit and a distinct α subunit.

Evidence that these three glycoproteins are related comes from the observation that patients with recurrent bacterial infections are deficient in all three of these proteins (Springer et al 1984). It was reported by Todd (1982) that a deficiency in Mo1 antigen on the surface of human granulocytes was associated with defects in the C3 and IgG dependent phagocytosis of these cells. Both the α and β subunits of the Mo1 antigen appeared to be deficient in these patients. In agreement with these observations. Dana et al (1984) found that patients with Mo1 deficiencies on both granulocytes and monocytes were defective in IgG and C3 dependent phagocytosis. Todd et al (1984) studied the subcellular location of the Mo1 antigen and found it to be primarily located in the specific granules, with only a small proportion present on the membrane. Stimulation of the granulocytes with chemotactic agents led to translocation of Mo1 from the specific granules to the plasma membrane resulting in a five- to ten-fold increase in surface expression. This increase was associated with an increase in surface adhesion of the neutrophils. Neutrophils from two patients who did not express the Mo1 antigen were defective in both adherence and chemotaxis. Arnaout et al (1984) showed that stimulation of the patients' granulocytes led to an increase in surface expression of Mo1 but this was still considerably less than normal. From these experiments it was concluded that Mo1 deficiency may be partly responsible for the functional abnormalities which lead to recurrent bacterial infection in these patients. Springer et al (1984) found that the entire family of glycoproteins, Mac1, LFA-1 and P150/95 were deficient in this group of patients, while CR1 was present in normal amounts. Recently monoclonal antibodies have been developed against P150/95 (Lanier et al 1985; Springer, Miller and Anderson 1986). The monoclonal antibody anti-leu M5 reacts with a specific epitope in the 150,00 dalton α subunit. Leu M5 is found to be expressed on monocytes and granulocytes but not blood lymphocytes. Patients who are defective in Mo1 and LFA-1 expression also fail to react with anti-leu M5. Springer et al (1986) have suggested that the primary defect in these patients is the failure to synthesise the common β subunits. It was proposed that while the α chains of the three proteins were synthesised normally they require to become associated with the β subunit before being transported to the cell surface and as the β subunit was not synthesised transportation did not occur.

1.34(b) Binding Site Properties of CR3

CR1, CR2 and CR3 all have binding sites for iC3b. The binding of iC3b to CR1 is weak and requires large amounts of fixed iC3b (Ross et al 1983). CR3 does not bind to any other C3 fragments apart from iC3b. Because of the possibility of iC3b binding to all three receptors, when studying the binding of CR3 it is necessary to ensure that CR1 and CR2 sites on the cell surface are preblocked with saturating amounts of anti-CR1 and anti-CR2 antisera. The binding of CR3 to iC3b resembles the binding of bovine serum conglutinin (K) to iC3b in that they both require the presence of carbohydrates and calcium ions. The sugar, N-acetyl-D-glucosamine (NADG) inhibits the binding of both K and CR3 to iC3b. Both K and CR3 binding is also inhibited by EDTA (Ross et al 1983). It was originally thought that CR3 might be the human homologue of bovine K but the observations of Davis and Lachmann (1983) which showed distinct structural differences between the two imply that this is not the case. Recently Ross et al (1985) showed that K like CR3 can bind directly to unopsonized yeast or zymosan particles. It was found that neutrophils treated with a monoclonal antibody against CR3, (Leu 15) could bind directly to yeast particles in the absence of fixed iC3b and trigger their ingestion. However these same neutrophils could not bind to EC3bi. When treated with the monoclonal antibody, OKMI, the reverse was true, binding to EC3bi was normal and no ingestion followed but binding to yeast particles was inhibited. The results were explained by suggesting that there are two binding sites in the $\alpha$ chain of CR3, one which can be blocked by Leu 15 and which is responsible for binding to fixed iC3b and one which can bind directly to yeast or zymosan without the presence of fixed iC3b and which triggers ingestion. This binding site can be blocked by OKMI.

This conclusion has been supported by Arnaout et al (1985) who also using monoclonal antibodies has shown that there are two functional domains in CR3. One of these domains is involved in binding iC3b and one is involved in inhibiting leukoaggregation, chemotaxis, spreading and zymosan induced $O^{2-}$ production. Ross et al (1985) have reported that the sugar to which both K and CR3 bind is $\beta$-glucan. Neutrophil binding to yeast particles could be inhibited by addition of soluble $\beta$-glucan but EC3bi rosettes were not affected by this $\beta$-glucan. This again implies the presence of two binding sites in CR3, one for yeast $\beta$-glucan and one for iC3b which is not inhibited by $\beta$-glucan.

1.34(c) Function of CR3

As has already been mentioned CR3 appears to be more effective than CR1 in promoting ingestion of iC3b opsonised particles (Wright and Silverstein 1982). CR3 and CR1 may also act in synergy to promote phagocytosis of C3b opsonised particles (Ross et al 1985) with CR3 being responsible for triggering ingestion. Ross et al (1985) have indicated that CR3 may be able to bind directly to unopsonised zymosan particles to promote their phagocytosis. Phagocytosis of unopsonised zymosan

results in the generation of a respiratory burst a feature which has not been found to be associated with phagocytosis of C3b coated particles (Newman and Johnston 1979). Even after stimulation of monocytes with PMA no respiratory burst was observed (Wright and Silverstein 1982). It therefore appears that CR3 may be more effective than CR1 in phagocytosis. This may be of particular importance in phagocytosis of particles opsonised via the alternative pathway. Particles which activate the alternative pathway have surfaces which favour the formation of a C3 convertase. This might be due to the lower affinity of regulatory proteins such as CR1 for fixed C3b on these surfaces. If this is the case then ingestion of opsonised particles which contain both iC3b and C3b is more likely to be mediated through the binding of iC3b to CR3.

1.35(a) Structure of CR4 and Binding Characteristics

Suggestion that a fourth complement receptor might exist arose from the observation that monocytes and neutrophils which were considered to lack CR2 bound to fluorescent microspheres coated with C3dg (Ross et al 1983). That the rosettes were due to contamination of the C3dg with iC3b or C3b was ruled out by the addition of EDTA which blocks CR3 activity and anti-CR1 which blocks CR1 activity. These observations suggested that perhaps phagocytic cells did possess a receptor similar to CR2, but the exact nature of this receptor was unknown. Frade et al (1985a) noted that neutrophils could form rosettes with E bearing over 45,000 molecules of C3dg. This rosetting was not due to the presence of CR2 as determined by the lack of uptake of $^{125}$I-labelled polyclonal or monoclonal anti-CR2 and by the inability of large excesses of anti-CR2 to inhibit C3dg rosettes. This receptor on phagocytic cells has tentatively been described as CR4. Its structure remains unknown although a report by Wright, Licht and Silverstein (1984) suggests that it could be the p150/95 antigen recognised by the monoclonal antibody IB4. IB4 immunoprecipitates CR3 and LFA-1 and an $\alpha$ chain of 153,000 daltons from cultured monocytes. Once stimulated with PMA cultured monocytes formed rosettes with EC3d. These stimulated monocytes were allowed to spread on surfaces coated with OKM10, which recognises the $\alpha$ chain of CR3, or on surfaces coated with TA-1, which recognises the $\alpha$ chain of LFA-1 or on surfaces coated with IB4. The nonadherent side of the monocytes which were spread on these various surfaces were then tested for their ability to rosette with EC3d. It was found that only spreading on IB4 abolished rosetting activity. They concluded from this that the CR2 like receptor was being bound by IB4 at the substrate attached portion leaving the apical portion of the membrane void of these receptors. Inada et al (1983b) also showed that unstimulated monocytes did not bind EC3d but required first to be cultured in the presence of foetal calf serum (FCS) whereupon they showed a progressive increase in ability to rosette with EC3d. Frade et al (1985a) found that rosettes did not form with E bearing over 100,000 molecules of C3d. They did however rosette with E bearing 45,000 molecules of C3dg. Vik and Fearon (1985) studied the binding of neutrophils to soluble rather than substrate-bound C3. In this way they were able to analyse the reversibility, saturability and

ligand specificity of the reaction. They found that uptake of $^{125}$I-labelled C3dg was saturable and specific with approximately 14,000 C3dg binding sites per cell at saturation. This number increased to 21,000 sites per cell when labelled dimeric C3dg was used. Uptake of labelled C3dg was competitively inhibited equally well with C3dg, C3d and iC3b and 20-fold less well with C3b. The ability of CR4 to bind to iC3b has also been suggested by the work of Frade et al (1985a) who showed that E bearing over 45,000 molecules were only partially inhibited from binding to neutrophils by the addition of anti-CR1 and anti-CR3.

1.35(b) Function of CR4

The function of CR4 is unknown. It may have a role in the binding of soluble complexes released from E bearing C3dg or iC3b. It is uncertain at the moment if CR4 is able to induce particle ingestion (Ross and Medof 1985).

1.36 Aims of this Study

When this study was begun in 1982 it was known that patients with SLE had reduced numbers of CR1 sites on their E but it was not known if this abnormality was acquired or inherited. Family studies conducted that year (Walport et al 1982) found that low CR1 numbers on E tended to cluster in families. This suggested that CR1 numbers on E were inherited but this hypothesis remained to be proved. Moreover, although it was known that reduced CR1 numbers on E was a general feature of the disease it was not known if this was also true of other cell types.

This study sought to determine the possible mechanism leading to the reduced number of CR1 sites on the E of patients with SLE. In addition the CR1 numbers on the neutrophils of SLE patients were quantified in an attempt to determine if reduced CR1 numbers in SLE patients were also evident on this cell type. The aims of this study were therefore (i) to purify CR1 and prepare an antibody to it, (ii) to compare the expression of E CR1 on identical and nonidentical twins in order to establish the relative roles of genetic and environmental factors in the regulation of E CR1 numbers in a normal population, (iii) to investigate the role of plasma and tissue enzymes and antigen/antibody complexes in the reduction of E CR1, (iv) to study CR1 on neutrophils from SLE patients to determine if this cell type also had reduced numbers of CR1, and finally, (v) to study the biosynthetic rates of CR1 in monocytes and lymphocytes from normal individuals and from patients with SLE.

# CHAPTER TWO

## 2.1 Introduction

The isolation of CR1 and the production of a polyclonal antiserum was a prerequisite for this project. Using the method described by Fearon (1979) a small quantity of CR1 had previously been isolated by Dr W S Kilpatrick. This purified CR1 was shown to be homogeneous by analysis on 5% SDS-PAGE gels which were run under reducing and nonreducing conditions. These revealed a single band of molecular weight 200,000 daltons when stained with 0.1% PAGE blue G-90. Antiserum raised to this preparation was shown to be specific by its ability to give a single line of identity with a crude CR1 preparation using double immunodiffusion in agarose gel and also by its ability to agglutinate human E, but not human E which had been trypsinised to remove CR1. As supplies of this original material were limited it became necessary to prepare a new batch.

Three attempts were made to purify CR1 from human membranes, only the last of which resulted in a homogeneous preparation of the protein. Each of the three attempts will be described and the modifications to the method which resulted from the experience gained over the course of the procedure will be discussed.

## Materials and Methods

### 2.2 Chemicals and Reagents

Chemicals and other reagents were obtained from the following companies:

Aldrich Chemical Company, Gillingham, Dorset

Benzamidine
Ethanolamine

Amersham International Buckinghamshire

$^{14}C$ Methylated Molecular Weight Markers

British Drug Houses (BDH) Chemicals Ltd, Poole, Dorset

All reagents were of Anlar quality

Acetic Acid
Agarose
Barbitone
Calcium Chloride
Copper Sulphate
D-Glucose
Dimethylsulphoxide (DMSO)
Dipotassium Hydrogen Phosphate
Disodium Hydrogen Phosphate
Ethylenediaminetetraacetic Acid (EDTA)
Gelatin
Glycine
Hydrogen Chloride
Magnesium Chloride
Methanol
Nonidet (NP-40)
Potassium Dihydrogen Phosphate
Potassium Iodide
Sodium Azide
Sodium Barbitone
Sodium Carbonate
Sodium Chloride
Sodium Dihydrogen Phosphate
Sodium Hydroxide
Sodium Potassium Tartarate
Trichloroacetic Acid
Tris(hydroxylmethyl)methylamine (Tris)

Sigma Chemical Company, Fancy Rd, Poole, Dorset

Acrylamide

Ammonium Persulphate
Alpha-Methylmannoside
N,N'-Methylene Bisacrylamide
Bovine Serum Albumin (BSA)
Chloramine T
Coomasie Blue
Cyanogen Activated Sepharose 4B
Dowex-1 (mesh 100-200)
Folin and Ciocalteu's Phenol Reagent
Glycerol
Lentil Lectin
2' Mercaptoethanol
High Molecular Weight (HMW) Markers
N N N,'N'-Tetramethylenediamine (TEMED)
Phenylmethanesulphonyl Fluoride (PMSF)
Sodium Dodecylsulphate (SDS)
Polyoxyethylenesorbitan monolaurate (Tween 20)
Tyrosine
Zymosan

Flow Laboratories, Irvine, Ayrshire

Sheep E in Alsever's solution

Difco Laboratories, PO Box 14B, Central Ave, Molsey, Surrey

Freund's Incomplete Adjuvant

Linbro, Flow Laboratories, Irvine, Ayrshire

Microtitre Plates

Luckman Ltd, Victoria Gds, Burgess, Hill, Sussex

LP3 Tubes

Scientific Instrument Centre Ltd, Unit 34D, Darham Dr, Eastleigh, Hampshire

Visking Tubing

Pharmacia House, Midsummer Boulevard, Milton Keynes

Cellophane Membrane

Amicon Corporation, Lexington, Mass 02173, USA

Amicon PM 10 Membrane

Amicon PM 30 Membrane

Bio-Rad Laboratories Ltd, Caxton Way, Watford, Hertfordshire

Biogel A5-M
Biorex 70 (mesh 200-400)
Silver stain kit

Millipore (UK) Ltd, Peterborough Road, Harrow, Middlesex

Pellicon Cassette.

Blood Transfusion Service, Law Hospital, Carluke

Units of fresh and expired packed red cells.

Isotope Dispensary, Western Infirmary, Glasgow

$^{125}I$ carrier free

2.3 Optical Density (OD) Readings

OD readings were measured on a Shimadzu spectrophotometer, Model No UV120-02.

2.4 Conductivity Measurements

Conductivity was measured at 0°C on a conductivity meter, Model CD M3 (Radiometer Copenhagen).

2.5 pH Readings

pH was measured on a pye model 292 pH meter (Pye Unicam).

2.6 General Methods

2.6(a) Preparation of Normal Rabbit Serum (NRS)

Rabbit blood was allowed to clot at 37°C for 30 min and then placed on ice for 30 min to allow the clot to retract. After centrifugation at 2000g for 5 min at 4°C the clear serum was separated from the clotted red cells. The serum was heat inactivated for 30 min at 56°C and stored at -20°C until required.

2.6(b) Dialysis

Visking tubing was boiled 3 times in EDTA (1 mmol $l^{-1}$) before being rinsed in deionised water. An appropriate length of tubing was cut and tied at one end, the open end was filled with the sample to be dialysed

and then tied firmly leaving no air spaces. The dialysis sac was then placed into the appropriate buffer.

2.6(c) Measurement of Protein Concentration by Folin Assay (Lowry et al 1951)

Buffers and Solutions

PBS (0.15 mol $l^{-1}$)

8g of sodium chloride, 1.21g of dipotassium hydrogen orthophosphate and 0.34g of potassium dihydrogen orthophosphate were mixed together in 1 litre of deionised water.

0.1%(w/v) BSA/PBS

0.1g of BSA was dissolved in 100mls of PBS.

Reagent A

Reagent A was prepared by dissolving 2g of sodium carbonate and 20mg of sodium potassium tartarate in 100mls of sodium hydroxide (0.1 mol $l^{-1}$).

Reagent B

Reagent B was a 0.15%(w/v) solution of copper sulphate in deionised water.

Reagent C

Reagent C was made up fresh daily by mixing 5mls of A with 100µl of B.

Reagent D

Reagent D consisted of a 1 in 2 dilution of Folin and Ciocalteu's phenol reagent in deionised water.

Procedure

Two millilitres of reagent C were added to a series of glass test tubes to which a 50µl aliquot of the sample to be tested was then added. After addition of 200µl of reagent D the tubes were mixed thoroughly and left to stand at room temperature for 30 min. The tubes were spun at 2000g for 5 min at 4°C and the $OD_{700nm}$ of the supernatant was measured. Controls included a background, to which 50µl of sample buffer had been added, and a set of standards which comprised of a series of tubes to which different quantities (5µg to 100µg) of a 0.1%(w/v) BSA/PBS solution had been added. The background reading was subtracted from all the other readings and the protein concentration determined by constructing a standard curve with the BSA concentration of the standards plotted

against the $OD_{700nm}$ (Fig 2.1). From this graph the protein concentration in the samples could be determined.

Measurement of Protein Concentration in Samples containing NP-40

As NP-40 in buffers interfered with direct spectrophotometer readings at $OD_{280nm}$ the protein content in such samples was measured by Folin assay. After the 30 min incubation at room temperature the tubes were centrifuged at 2000g for 5min at room temperature and the clear supernatant was removed from the precipitated NP-40. The $OD_{700nm}$ of the supernatant was then measured.

2.6(d) Radioiodination of Anti-CR1 IgG by the Chloramine T Method (McConahey and Dixon 1966)

Reagents

Rabbit anti-CR1 IgG

Rabbit anti-CR1 IgG had previously been prepared by Dr W S Kilpatrick.

Buffers and Solutions

0.1%(w/v) BSA/PBS

0.1g of BSA were dissolved in 100mls of PBS.

Chloramine T

5mg of chloramine T were dissolved in 1ml of PBS.

L-Tyrosine

0.5mg of L-tyrosine were dissolved in 1ml of sodium hydroxide ($0.1$ mol $l^{-1}$).

10%(w/v) BSA/PBS

10g of BSA were dissolved in 100mls of PBS.

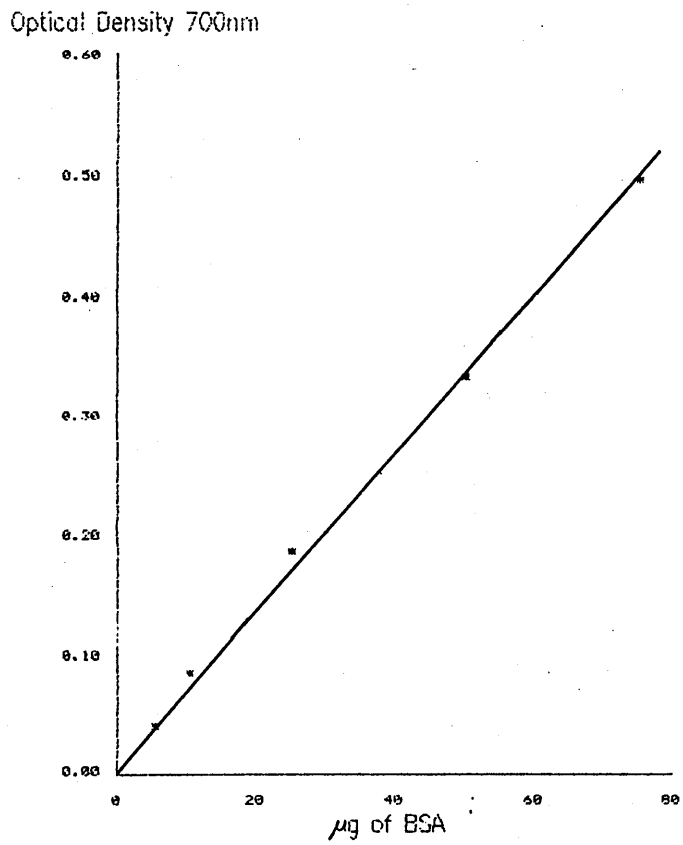
20%(w/v) TCA

20g of TCA were dissolved in 100mls of deionised water.

Fig 2.1 BSA Standard Curve

Legend

A linear standard curve for BSA was constructed by plotting the $OD_{700nm}$ of a known concentration of BSA against that concentration.


Optical Density 700nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
20
40
60
80
μg of BSA

Chromatographic Material

Preparation of Dowex Column

Dowex, an anion exchange resin, was washed 3 times in 0.1%(w/v) BSA/PBS before pouring into a 10ml glass pipette. The column was washed with this buffer until it had equilibriated.

Radioiodination

Procedure

Anti-CR1 IgG (1mg/ml) was dialysed for 3 hours against 5 litres of PBS at 4°C. One millilitre of this solution was placed into a glass vial containing 1 mCi (37MBq) of carrier free $^{125}I$. After the contents were mixed thoroughly, 10µl of a freshly prepared solution of chloramine T were added and mixed for 90 seconds. The reaction was stopped by the addition of 50µl of L-tyrosine. To separate the protein which had bound $^{125}I$ from the unbound $^{125}I$ the solution was passed over the Dowex column. One millilitre fractions were collected and screened by counting a 1:100 dilution of each in an automatic gamma (γ) counter. An elution profile was drawn by plotting the cpm in each fraction against the fraction number (Fig 2.2). Fractions containing the highest counts were pooled as indicated and analysed by trichloroacetic acid (TCA) precipitation to determine the percentage of radiolabelled protein to free $^{125}I$.

2.6(e) Acid Precipitation of Protein

Two hundred microlitres of a 10%(w/v) BSA/PBS solution were added to a microcap centrifuge tube and 10µl of a 1:100 dilution of the pooled fractions were added followed by 1ml of 20%(w/v) TCA solution. The tube was mixed thoroughly and centrifuged at 10,000g for 5 min at room temperature. The supernatant was decanted into a glass tube and the pellet was cut from the bottom of the microcap tube and placed into a second glass test tube. The $^{125}I$ content of the supernatant and the pellet were determined using an automatic γ counter. The percentage of TCA-precipitable radioactivity was determined according to the following formula:

$$\frac{\text{CPM in pellet} - \text{CPM in background (Bg)}}{(\text{cpm in pellet}-\text{cpm in Bg})+(\text{cpm in supernatant}-\text{cpm in Bg})} \times 100$$

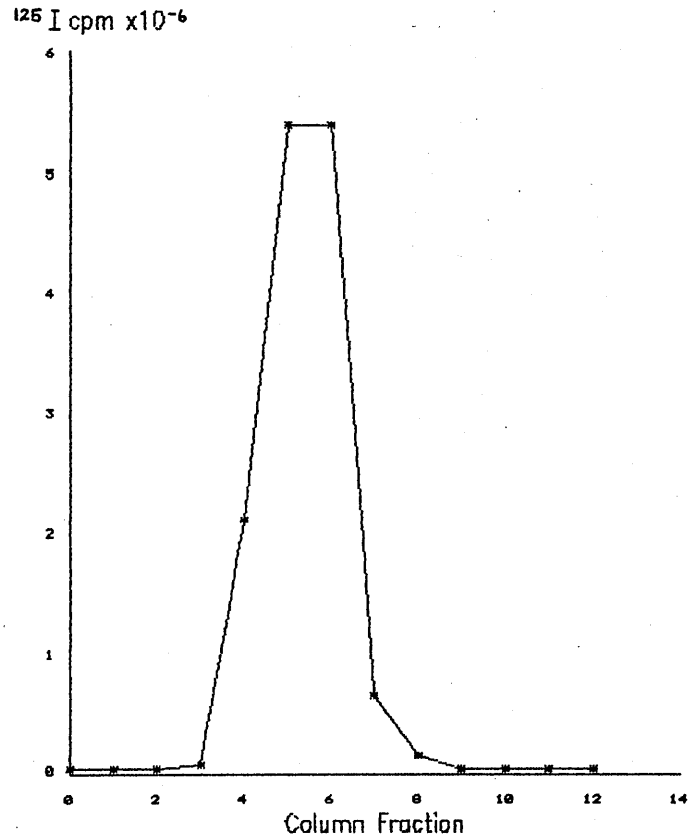
The background was determined by adding 10µl of PBS to the microcap tube instead of the labelled sample, following by treatment in the same way as the sample.

If the TCA precipitation was less than 85% the label was not used. The efficiency of labelling was determined by expressing the number of counts

Fig 2.2 Purification of $^{125}$I-Labelled anti-CR1 IgG from Free Iodine

Legend

Purification of $^{125}$I-labelled anti-CR1 IgG from free iodine using a 10ml Dowex column. Fractions(500µl) were collected and the amount of $^{125}$I-cpm in a 1:100 dilution of each fraction was plotted against the fraction number. The peak contains $^{125}$I-bound to anti-CR1 IgG as determined by acid precipitation with TCA. Fractions 5 and 6 were pooled.


125 I cpm x10-6
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Column Fraction

in the pooled fractions as a percentage of the total counts put over the column.

The protein concentration of the pooled fractions was obtained by measuring the $OD_{280}$ of the pool and subtracting the $OD_{280}$ of the 0.1%(w/v) BSA/PBS solution from the $OD_{280}$ of the pooled fractions.

2.6(f) Double Immunodiffusion in Agarose Gels (Ouchterlony 1958)

Buffers

Barbitone Buffer

9.21g of barbitone, 51.44g of sodium barbitone and 5g of sodium azide were dissolved separately in hot deionised water before being mixed together and made up to a final volume of 5 litres.

Preparation of Agarose Gel

Agarose (5g) was added to 500mls of hot barbitone buffer. When dissolved 15g of polyethylene glycol (PEG) with a molecular weight of 4,000 daltons were added.

Procedure

Hot agarose gel (5ml) was poured onto a glass slide which had been washed, swabbed with alcohol and placed onto a levelling tray. Once set wells were punched in the gel using a template (Fig 2.3). The agarose plugs were removed by vacuum pump suction and a sufficient volume of antiserum to fill each peripheral well was added. CR1 (32µg/ml) was added to the central well and the plate was left for 48 hours in a humid atmosphere to allow precipitation lines to develop.

Staining Ouchterlony Plates

Buffers and Solutions

Half Normal Saline

0.45g of sodium chloride were dissolved in 1 litre of deionised water.

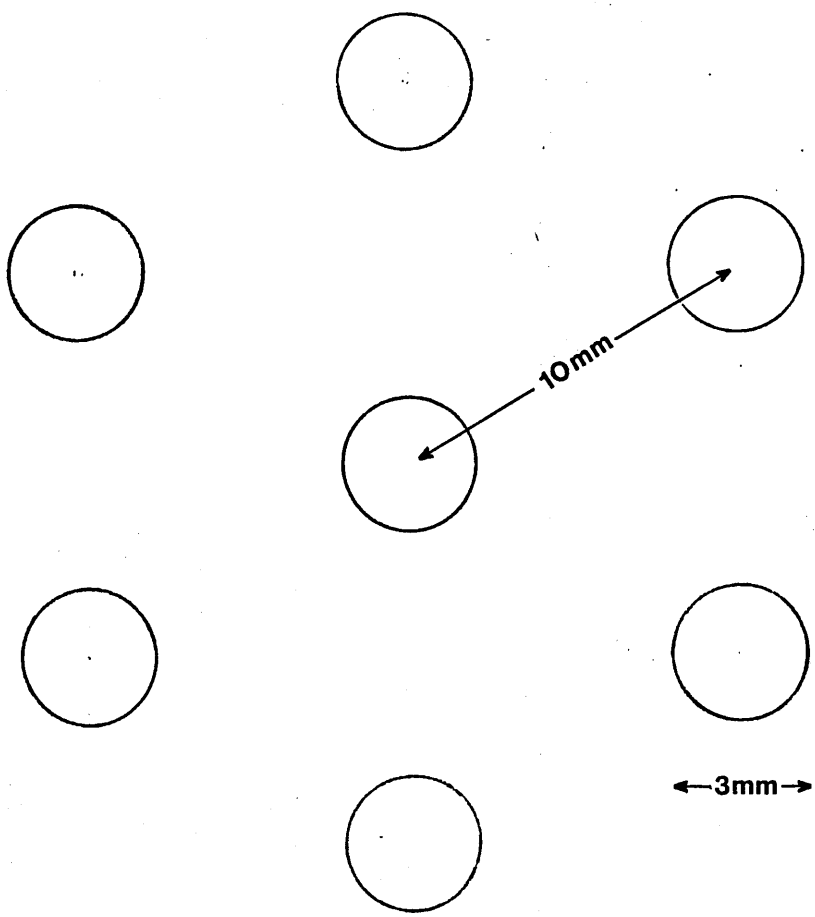
0.1% Coomassie Blue Solution

A methanol: acetic acid: water solution was made up in the proportions 1:1:8 (v/v/v). To 100mls of this solution 0.1g of coomassie blue were added.

Fig 2.3 Template for Ouchterlony Plate

Legend

Each well is 3mm in diameter and there is a distance of 10mm between the centre point of the central well and the centre of the peripheral wells.


10mm
3mm

Destain Solution

A methanol: acetic acid: water solution was made up in the proportions 1:1:8 (v/v/v).

Procedure

The plate was washed extensively in half normal saline for 1 day and then in deionised water for 1 day. The plate was dried overnight between sheets of filter paper. When dried, the plate was soaked for 2 to 3 min in 0.1% coomassie blue solution after which it was destained by soaking overnight in destain solution.

2.6(g) Preparation of Anti-CR1 Antiserum

One millilitre of purified CR1 (32μg/ml) plus 1ml of PBS were emulsified in an equal volume of Freund's complete adjuvant. Two millilitres of this solution were injected subcutaneously into 2 rabbits. This primary immunisation was followed by 2 weekly injections of the same quantity of CR1 until the antiserum obtained by test bleeds was assessed to be specific for CR1 by double immunodiffusion in agarose gels.

2.6(h) Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli 1970)

Buffers and Solutions

Electrophoresis Buffer

10mls of 10%(w/v) SDS, 33mls of Tris (0.75 mol $l^{-1}$) and 220mls of glycine (0.86 mol $l^{-1}$) were mixed together in a total volume of 1 litre in deionised water.

Sample Buffer(reducing)

3mls of 10%(w/v) SDS, 0.85mls of Tris (0.75 mol $l^{-1}$), 1ml of glycerol, 150μl of bromophenol blue (0.1% w/v), 0.5ml 2-mercaptoethanol and 5mls of deionised water were mixed together.

Acrylamide/Bisacrylamide Solution

30g of acrylamide and 0.8g of bisacrylamide were made up to 100mls with deionised water and stored at 4°C for up to 4 weeks.

10% Gelatin Solution

10g of gelatin were dissolved in 100mls of deionised water by heating in a pressure cooker this solution was then stored at 4°C. Prior to use the gelatin solution was desolidified by placing in boiling water.

Coomassie Blue Stain Solution

A methanol: acetic acid water solution was made up in the proportions 5:1:4 (v/v/v), 0.1% (w/v) coomassie blue was then dissolved in this.

Destain Solution

Destain solution was made up as previously described in section 2.6(f).

Gel Recipes

Separating Gel

| % Gel | Acrylamide/ Bisacrylamide | Water (ml) | Tris (0,75 mol $l^{-1}$ pH 8,8) (ml) | 10% SDS(µl) | TEMED (µl) | Ammonium/Per-Sulphate (mg) |
|---|---|---|---|---|---|---|
| 5 | 10 | 19,4 | 30 | 0,6 | 30 | 40 |
| 7½ | 10 | 9,6 | 20 | 0,4 | 20 | 60 |

Stacking Gel

| % Gel | Acrylamide/ Bisacrylamide | Water (ml) | Tris (0,75 mol $l^{-1}$ pH 8,8) (ml) | 10% SDS(µl) | TEMED (µl) | Ammonium/Per-Sulphate (mg) |
|---|---|---|---|---|---|---|
| 3 | 1,8 | 13 | 3 | 0,18 | 9 | 18 |

Procedure

Slab gels were poured between glass plates 20cmx20cm separated by 1mm spacers and sealed with 10% gelatin. The separating gel was poured first and allowed to polymerise for 1 hour at room temperature with a layer of water carefully added to the surface to prevent evaporation. After setting the water was removed carefully and the stacking gel was poured. A spacing comb was placed into the stacking gel prior to setting to allow formation of lanes. The gel was then clipped into the electrophoresis tank which had been filled with electrophoresis buffer.

2.6(i) Preparation of Samples for SDS-PAGE

To 40µl of sample, 40µl of sample buffer were added and the samples boiled for 2 min. The reduced samples were then layered carefully into the individual lanes using a micropipette and electrophoresed for 3 hours

at 35mA at room temperature. Once the electrophoresis was complete the gel was treated by staining with coomassie blue or the silver impregnation technique.

2.6(j) Coomassie Blue Staining

The gel was fixed and stained for 1 hour at 37°C in stain and then destained overnight in destain solution at 37°C. This type of staining does not detect proteins with a concentration of less than 0.2μg and therefore some gels were silver stained as this is a more sensitive method of staining being able to detect 2- to 5-fold lower protein concentrations than coomassie blue staining.

2.6(k) Silver Staining

Gels were silver stained using a silver stain kit. After the protein in the gel had been fixed for 30 min in 400mls Of 40% methanol/10% acetic acid(v/v), the gel was washed twice in 10% ethanol/5% acetic acid (v/v)(15 min each wash). This was followed by addition of 200mls of oxidising solution for 5 min and then by sufficient washes in deionised water to ensure that all colour was removed from the gel. The silver reagent (200mls) was added and the incubation continued for 20 min after which a quick wash in deionised water was followed by the addition of the developing reagent. The reaction was stopped by the addition of 5%(v/v) acetic acid once the protein bands had reached the desired intensity.

2.6(l) Determination of Molecular Weight

The molecular weight of unkown protein samples was determined by running on the same gel a series of proteins of known molecular weight from which a standard curve could be constructed (Fig 2.4). In the case of gels stained with coomassie blue nonradioactive high molecular weight markers were used and $^{14}C$ methylated molecular weight markers were used if the gel was to be subjected to autoradiography. By calculating the relative mobility (Rf valve) of the unknown sample, which is equivalent to:

$$\frac{\text{the distance travelled by the protein}}{\text{the distance travelled by the dye front}}$$

the molecular weight of the protein could be determined from the standard curve.

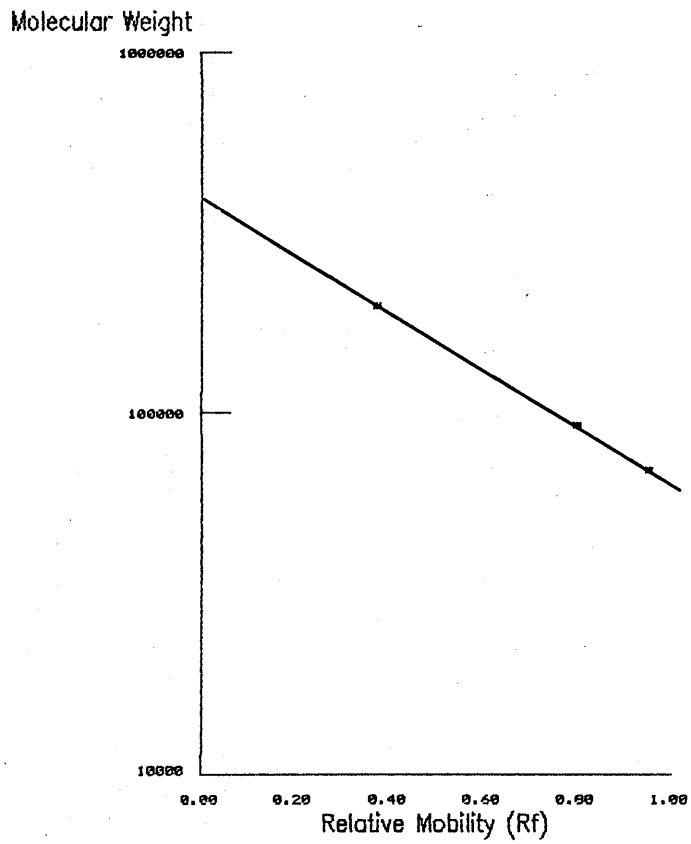
2.7 Screening Columns for CR1

Over the course of the 3 CR1 preparations 3 different methods were used for screening the columns for CR1 activity, radioimmunoassay, agglutination of EAC43b and inhibition of alternative pathway C3 convertase activity.

Fig 2.4 Molecular Weight Standards

Legend

A standard curve was constructed by plotting the known molecular weight of a series of proteins against the relative mobility (Rf value) of that sample. The proteins used in the construction of this curve were BSA (molecular weight 69,000 daltons), phosphorylase b (molecular weight 92,000 daltons) and myosin (molecular weight 200,000 daltons).


Molecular Weight
1000000
100000
10000
0.00
0.20
0.40
0.60
0.80
1.00
Relative Mobility (Rf)

2.7(a) Radioimmunoassay For Screening Columns (Iida et al 1982)

Reagents

C3

C3 was purified by Dr E Holme. It was diluted in PBS to a final concentration of 50μg/ml.

Anti-CR1 IgG

Anti-CR1 IgG had previously been prepared by Dr W S Kilpatrick and was radiolabelled as described in (2.6(d)).

Buffers

Blocking Buffer

1g of BSA was dissolved in 100mls of PBS/BSA.

Wash Buffer

PBS/BSA containing 0.005%(v/v) Tween 20.

Procedure

Fifty microlitres of purified C3 (50μg/ml) were added to plastic LP3 tubes and the tubes incubated for 2 hours at room temperature. The C3 was then removed by vacuum suction and the tubes washed 3 times in blocking buffer after which 100μl of blocking buffer were added and the tubes left to incubate overnight at room temperature to allow blockage of free binding sites. After removal of the buffer, 25μl of test sample or 25μl of diluted purified CR1 (1:5 to 1:20) were added and the tubes incubated at 37°C for 2 hours to allow binding of CR1 to C3 after which the sample was removed and the tubes washed 3 times with wash buffer. Twenty five microlitres of $^{125}I$ anti-CR1 IgG (500μg/ml) diluted in blocking buffer were added and incubation continued for 1 hour at room temperature. Finally the label was removed and the tubes washed 4 times in wash buffer. The amount of bound $^{125}I$ anti-CR1 IgG was measured as cpm by counting the plastic tubes in an automatic γ counter. The background from a control tube containing 25μl of PBS instead of sample material was subtracted from each reading to give the specific cpm for each sample.

Standard Curve for CR1

A small quantity of CR1 had previously been purified by Dr W S Kilpatrick but as there was an insufficient volume of the material to allow an accurate estimation of protein content the assay could not be standardised in terms of its sensitivity to detect CR1. However, the

purified CR1 was used at a 1:5 to 1:20 dilution to form a standard curve for the RIA (Fig 2.5). There was a linear relationship between the titre of CR1 and the number of cpm in the assay. For this reason the total number of cpm in a sample was taken as an estimate of the amount of CR1 in the sample assuming that the CR1 present had not lost any of its activity.

2.7(b) Agglutination of EAC43b

The presence of CR1 in a sample can be detected by its ability to agglutinate EAC43b. The concentration of CR1 present is indicated by the titre of sample at which haemagglutination is obtained.

Reagents Anti-sheep E stroma Ig$\mathcal{M}$ antibody was prepared by Professor K Whaley.

Buffers and Solutions

5xVBS (Veronal 5x buffer)

85g of sodium chloride and 3.75g of sodium barbitone were mixed together in 1 litre of deionised water. This was then heated until almost boiling whereupon 5.57g of barbitone were added and the solution made up to 2 litres with deionised water.

1xVBS (Isotonic VBS)

A 1:5 dilution of 5xVBS was made in deionised water.

GVB$^{2-}$

10mls of a 10% gelatin solution (10g of gelatin per 100 mls of deionised water) were added to 200mls of 5xVBS which was then made to 1 litre with deionised water.

GVB$^{2+}$

10mls of a 10% gelatin solution were added to 200mls of 5xVBS after which 5mls of calcium chloride (0.03 mol $l^{-1}$) and 10mls of magnesium chloride (0.1 mol $l^{-1}$) were added and the solution made to 1 litre with deionised water.

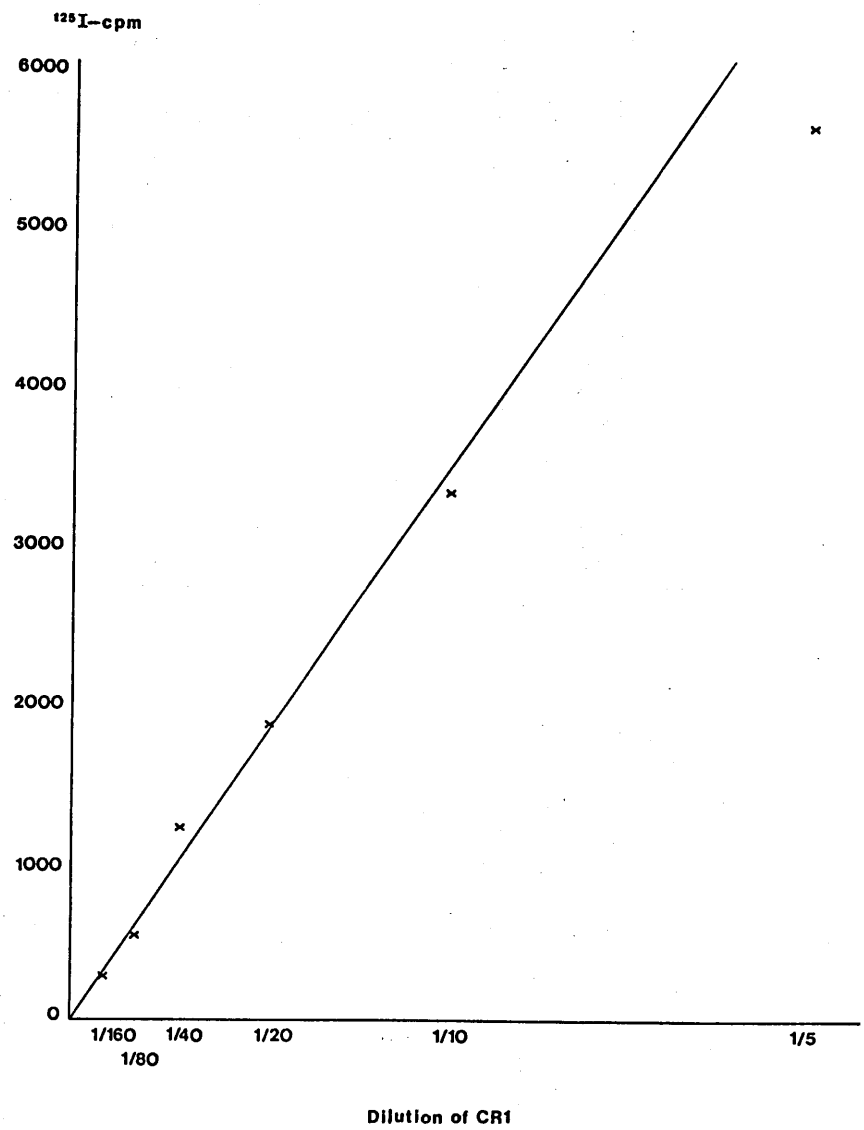
D5W$^{2+}$

50g of D-glucose were dissolved in 750mls of deionised water after which 5mls of calcium chloride (0.03 mol $l^{-1}$) and 10mls of magnesium chloride (0.1 mol $l^{-1}$) were added and the solution made to 1 litre with deionised water.

Fig 2.5 CR1 Standard Curve

Legend

A linear standard curve for purified CR1 was constructed by plotting the $^{125}$I-cpm of a known dilution of CR1 against that dilution.


$^{125}$I-cpm
6000
5000
4000
3000
2000
1000
0
1/160
1/80
1/40
1/20
1/10
1/5
Dilution of CR1

DGVB $^{2+}$

An equal volume of D5W$^{2+}$ and GVB$^{2+}$ were mixed together.

EDTA GVB$^{2-}$ (0.04 mol $l^{-1}$)

230mls of EDTA (0.086 mol $l^{-1}$) pH 7.4 were mixed with 200mls of GVB$^{2-}$.

Isotonic EDTA (0.086 mol $l^{-1}$ pH 7.4)

22.3g of EDTA were dissolved in 500mls of deionised water to make a 0.12 mol $l^{-1}$ EDTA solution. 3.6g of sodium hydroxide were dissolved in 300mls of water to make a 0.3 mol $l^{-1}$ solution, 500mls of EDTA (0.12 mol $l^{-1}$) were then mixed with 185mls of sodium hydroxide (0.3 mol $l^{-1}$) to result in a pH of 7.4.

Normal Saline

0.9g of sodium chloride were dissolved in 100mls of deionised water.

Preparation of EAIgMC43b for Agglutination Assay

Sheep E in Alsever's solution were centrifuged at 2°C for 5 min at 2000g. The supernatant, plasma and buffy coat were removed by aspiration. The cells were then washed 3 times in EDTA GVB$^{2-}$ (0.01 mol $l^{-1}$) and resuspended in this buffer. One hundred microlitres of the E suspension was added to 2.9mls of deionised water and the OD was measured on a spectrophotometer at 541nm wavelength ($OD_{541nm}$). An OD reading of 0.385 corresponds to a sheep E concentration of $1x10^9$/ml (Whaley 1985). Thus the E were adjusted to $1x10^9$/ml using the formula:

$$\text{Vol E required} = \text{Initial volume} \quad X \quad \frac{OD_{541} \text{ Measured}}{0.385}$$

EA

Anti-sheep E stroma IgM antibody was titrated to find the maximum subagglutinating dose. Fifty microlitres of PBS were added to the wells of a microtitre plate, 50µl of the neat rabbit anti-sheep IgM was then added to the first well and serially diluted out to 1: 2000. Sheep E (50µl $1x10^9$/ml) were added to each well and the plate was mixed and left to stand at room temperature for 1 hour and the haemagglutination pattern observed. The lowest dilution of antibody which did not cause agglutination was used for the preparation of antibody sensitised sheep E (EA). The maximum subagglutinating concentration of IgM antibody diluted in 0.01M EDTA GVB$^{2-}$ (0.01 mol $l^{-1}$) was warmed to 37°C and added to pre-warmed E ($1x10^9$/ml) and the mixture continually shaken for 30 min at 37°C. The EA were centrifuged at 2,000g for 5 min at 2°C and washed once

in $GVB^{2-}$ EDTA (0.01 mol $l^{-1}$) and twice in $GVB^{2+}$ and resuspended to $1x10^{8}$/ml in $GVB^{2+}$.

Preparation of R3

One hundred milligrammes of zymosan were suspended in 10mls of isotonic VBS and boiled for 30 min then centrifuged at 2000g for 5 min at room temperature. The pellet was washed twice in isotonic VBS and resuspended in 10mls of isotonic VBS. One millilitre of this zymosan (10mg/ml) was put into a test tube and centrifuged at 2000g for 5 min at room temperature. Ten millilitres of fresh NHS were added to the pellet and the mixture incubated for 1 hour at 37°C. The zymosan was centrifuged at 2000g for 5 min at 2°C and the supernatant which contained R3 was decanted and stored in aliquots at -70°C.

Preparation of EAC43

Nineteen millilitres of EAIgM ($2x10^{8}$/1ml) were warmed to 37°C. One millilitre of R3 pre-warmed to 37°C was added to the EA in a shaking water bath. Incubation was continued for 75 seconds whereupon 200µl of pre-warmed antrypol (100mg/ml) were added and incubation continued for a further 2 min. The cells were then diluted in an equal volume of ice-cold EDTA (0.01 mol $l^{-1}$) $GVB^{2-}$ and centrifuged at 2000g for 5 min at 2°C then resuspended in this buffer and incubated for 2 hours at 37°C to decay off the C2, after which the cells were again centrifuged at 2000g for 5 min at 2°C, resuspended in $DGVB^{2+}$, washed once and finally resuspended to $1x10^{8}$/ml in $DGVB^{2+}$.

Procedure

One hundred microlitres of PBS were added to each well of a microtitre plate. Five microlitres of test sample were added to the first well and serially diluted out to 1:2500. Fifty microlitres of EAC43 ($1x10^{8}$/ml) were then added to each well and the plate was shaken and left to stand at room temperature for 3 hours to allow the cells to sediment. The pattern of haemagglutination was then noted. Control wells had no test sample added and were always haemagglutination negative.

2.7(c) Inhibition of Alternative Pathway C3 Convertase Activity

CR1 has been reported to accelerate the decay of C3bBbP, the alternative pathway C3 convertase (Fearon 1979; Iida and Nussenzweig 1981). The presence of CR1 can be measured by determining the ability of a sample to inhibit the lysis of EAC43bBbP.

Reagents

C Rat

Rat serum was used at a 1:15 dilution in EDTA (0.04 mol $l^{-1}$) $GVB^{2-}$.

Factors B, D and P

Factors B, D and P were kindly donated by Professor K Whaley. The optimal concentrations of these factors had been predetermined by titration of each one individually.

EAC43bBb

EAC43bBb were prepared by incubating an equal volume of EAC43b($1x10^8$/ml) in $DGVB^{2+}$ with $DGVB^{2+}$ containing the appropriate dilutions of B, D and P for 30 min at 30°C. The EAC43bBbP were centrifuged at 2000g for 5 min at 2°C, the supernatant was decanted and the wells washed once in ice-cold EDTA (0.01 mol $l^{-1}$) $GVB^{2-}$ and resuspended in this buffer at $1x10^8$/ml

Procedure

Samples to be tested were diluted 1:25 in EDTA (0.01 mol $l^{-1}$) $GVB^{2+}$. EAC43bBbP (100µl) were incubated with 100µl of the diluted sample for 15 min at 30°C to allow CR1 in the sample to accelerate the decay of the C3 convertase. C rat (300µl) was added to each tube to supply the terminal components in order to develop haemolytically active sites. Incubation was continued for 1 hour at 37°C during which lysis of E occurred, the extent depending on the amount of convertase activity remaining on the cells. A series of control tubes were set up the contents of which are shown below:

| | Materials Added | | | |
|---|---|---|---|---|
| Controls | 0.01M EDTA/$GVB^{2-}$ | EAC43bBbP | EAC43 | C-Rat |
| Reagent Blank | 100µl | - | 100µl | 300µl |
| Cell Blank | 400µl | 100µl | - | - |
| Complement Colour | 200µl | - | - | 300µl |
| 100% Lysis | 100µl | 100µl | - | 300µl |
| Solo | 100µl | 100µl | - | 300µl |

The reaction was stopped after 1 hour by the addition of 2mls of saline to each tube except the 100% lysis control to which 2mls of deionised water were added instead. The tubes were then centrifuged at 1000g for 5 min at 4°C and the $OD_{414}$ was read on a spectrophotometer.

Calculation of results

The results were calculated according to the formula described by Gigli, Ruddy and Austen (1968) which is shown below:

$$Z' = -\ln \frac{OD_{414} \text{ inhibitory sample} - OD_{414} \text{ reagent blank}}{OD_{414} \text{ solo} - OD_{414} \text{ reagent blank}}$$

Where Z' is a measure of the degree of inhibition of lysis.

2.8 Buffers for CR1 Purification

Phosphate Buffers

A stock solution of disodium hydrogen phosphate (0.1 mol $l^{-1}$) and a stock solution of sodium dihydrogen phosphate (0.1 mol $l^{-1}$) were prepared. These 2 solutions were added together until the desired pH was obtained and then diluted to give the appropriate molarity.

Wash Buffer

9g of sodium chloride were dissolved in 1 litre of deionised water.

Lysis Buffer

Sodium phosphate buffer (5 mmol $l^{-1}$ pH 7.8) containing PMSF (0.5 mmol $l^{-1}$) and 0.02%(w/v) sodium azide.

Solubilisation Buffer

Sodium phosphate buffer (5 mmol $l^{-1}$ pH 7.5) containing sodium chloride (0.15 mol $l^{-1}$), 1%(v/v) NP-40, PMSF (0.5 mmol $l^{-1}$) and 0.02%(w/v) sodium azide.

Biorex Equilibriating Buffer

Sodium phosphate buffer (0.05 mol $l^{-1}$ pH 7.2) containing sodium chloride (0.02 mol $l^{-1}$) and 0.2%(v/v) NP-40.

C3-Sepharose Equilibriating Buffer

Phosphate buffer (0.01 mol $l^{-1}$ pH 7.5).

Biogel Equilibriating Buffer

1 x VBS, prepared as described in chapter 2.7(b), containing 0.1%(v/v) NP-40.

Lentil Lectin Equilibriating Buffer

Tris (0.01 mol $l^{-1}$) hydrogen chloride (Tris HCl) pH 7.4 containing sodium chloride (0.2 mol $l^{-1}$), calcium chloride (0.7 mmol $l^{-1}$), manganous chloride (0.7 mmol $l^{-1}$), magnesium chloride (0.7 mmol $l^{-1}$) and 0.1%(v/v) NP-40.

These buffers were used in the first and second CR1 purification attempts. In the third purification the following inhibitors were added to the afore mentioned buffers, benzamadine (0.01 mol $l^{-1}$), PMSF (0.5 mmol $l^{-1}$), 0.02%(w/v) sodium azide, and isotonic EDTA (0.5 mmol $l^{-1}$ pH 7.4). In addition these inhibitors were added to the water which was used to dilute material before application to the chromatography columns.

## 2.9 Preparation of Chromatographic Material

### 2.9(a) Biorex

Biorex, a cation exchange resin, was equilibriated in 5 litres of equilibriating buffer, degassed and poured into a column (5x30cm). The column was equilibriated at 4°C by the passing through of 5 column volumes of equilibriating buffer. When the conductivity at 0°C and the pH of the eluate buffer were the same as the equilibriating buffer the column was ready for use.

### 2.9(b) Cyanogen Bromide (CNBr) Activated Sepharose 4B-C3

Reagents

C3

Purified C3 was kindly donated by Dr E Holme.

Buffers used in the Preparation of Cyanogen Bromide Activated Sepharose 4B-C3

Sodium Carbonate Buffer (Coupling Buffer)

A stock solution of sodium carbonate (1 mol $l^{-1}$) and a stock solution of sodium hydrogen carbonate (1 mol $l^{-1}$) were prepared and mixed together until the desired pH of 9 was obtained. After dilution to give the appropriate molarity, 0.1 mol $l^{-1}$, sodium chloride (0.5 mol $l^{-1}$) was added.

Acetate Buffer

A stock solution of sodium acetate (1 mol $l^{-1}$) and a stock solution of acetic acid (1 mol $l^{-1}$) were prepared and mixed together until a pH of 4

was obtained. This solution was diluted to give a 0.1 mol $l^{-1}$ solution after which sodium chloride (1 mol $l^{-1}$) was added.

Borate Buffer

A stock solution of boric acid (1 mol $l^{-1}$) and a stock solution of sodium tetraborate (1 mol $l^{-1}$) were prepared and mixed together to obtain a pH of 8. This was then diluted to give a 0.1 mol $l^{-1}$ solution after which sodium chloride (1 mol $l^{-1}$) was added.

Procedure

CNBr-activated Sepharose 4B (6g) was washed in a Buchner funnel for 15 min with HCl (1 mmol $l^{-1}$) and then mixed with C3 (7mg/ml) in coupling buffer. The Sepharose beads and C3 were mixed end over end on a Matburn mixer for 2 hours at room temperature, centrifuged at 2000g for 5 min and then resuspended and washed 3 times in coupling buffer. Any remaining active groups on the Sepharose were blocked by exposure to an equal volume of ethanolamine (1 mol $l^{-1}$ pH 8.0) and then mixed end over end for 2 hours at room temperature. The beads were then washed in acetate buffer and then in borate buffer. This washing cycle was repeated 3 times before the beads were finally resuspended in C3-Sepharose equilibriating buffer, poured into a 10ml syringe barrel and washed with a sufficient quantity of buffer to equilibriate the column.

2.9(c) Biogel-A-5M

Biogel was equilibriated in VBS containing 0.1% NP-40 degassed and poured into a column (2.6x90cm). This column was equilibriated by passing 1 column volume of equilibriating buffer through the column.

2.9(d) Lentil Lectin-Sepharose

The Sepharose beads were washed as previously described and mixed with 20mg of lentil lectin in coupling buffer plus α methylmannoside (0.1 mol $l^{-1}$). The beads were rotated end over end at 4°C overnight and then washed in coupling buffer 3 times before being mixed with ethanolamine (1 mol $l^{-1}$ pH 8.0) for 2 hours at room temperature. This was followed by 3 cycle washes with acetate and borate buffer before resuspension in lentil lectin equilibriating buffer. The beads were then poured into a 1ml Pasteur pipette which had been plugged with glass wool and washed with 10mls of equilibriating buffer.

2.10 Purification of CR1 from Erythrocyte Membranes (1st and 2nd Attempts)

Ten (500ml) aliquots of expired blood were centrifuged at 4°C at 2000g for 10 min. The plasma and buffy coat were removed and the cells were washed 3 times in wash buffer. The packed red cells were then lysed by stirring overnight at 4°C in 15 litres of lysis buffer. The lysed

membranes were centrifuged in a Beckman Ultracentrifuge model L2-65B at 50,000g for 30 min at 4°C and the membranes collected as a flocculent pellet. The membranes were washed in 9 litres of lysis buffer by continual centrifugation at 50,000g for 30 min until they were free of haemoglobin after which they were suspended overnight at 4°C in 1.5 litres of solubilisation buffer. The solubilised membranes were centrifuged at 50,000g for 30 min at 4°C and the supernatant collected.

The supernatant was diluted with sufficient ice-cold water to lower the conductivity to below 6mS at 0°C and applied to the equilibriated Biorex column by means of a peristaltic pump at a constant flow rate of 100mls per hour. The insoluble Biorex particles contain mobile positively charged ions on the surface and these ions exchange with positively charged molecules (such as CR1) in the applied sample. These molecules were eluted by application of a linear salt gradient of 500mls with the limit buffer containing sodium chloride (0.6 mol $l^{-1}$). Ten millilitre column fractions were collected and screened for CR1 activity by R1A. The fractions containing CR1 activity were pooled and concentrated by positive pressure ultrafiltration using an Amicon PM-30 membrane.

This pool was diluted with 600mls of ice-cold water to lower the conductivity to below 6mS and applied at 4°C to the C3-Sepharose column. This column was washed through with equilibriating buffer and then sequentially with equilibriating buffer containing 0.1% NP-40, equilibriating buffer containing sodium chloride (0.2 mol $l^{-1}$) and equilibriating buffer. These washes were to ensure the removal of molecules bound weakly by both hydrophobic and charge interactions to the C3-Sepharose. Elution of solubilised CR1 from the C3-Sepharose followed application of equilibriating buffer containing both 0.1% NP-40 and sodium chloride (0.2 mol $l^{-1}$). Ten millilitre fractions were collected and screened in a similar way to the Biorex column. Those with CR1 activity were pooled and concentrated using an Amicon PM-30 membrane.

Five millilitres of the concentrated pool were carefully layered onto the Biogel column. Fractions (3mls) were collected and screened by R1A. Those with greatest CR1 activity were pooled and concentrated using an Amicon PM-10 membrane.

This pool was then added to the lentil lectin column. CR1 has a carbohydrate moiety which binds to the lentil lectin and which can be eluted by addition of equilibriating buffer containing $\alpha$ methylmannoside (0.2 mol $l^{-1}$) which has a stronger affinity for the lentil lectin than CR1.

## 2.11 Washing Erythrocyte Membranes with the Pellicon Cassette System

Eighteen packs of mixed fresh and expired blood were washed 3 times in wash buffer and lysed overnight at 4°C in 7 litres of lysis buffer. The lysates were washed free of haemoglobin in the Pellicon cassette. This apparatus allowed the E ghost membranes to be passed over a 0.45$\mu$M

Durapore filter cassette, which retained the membranes while allowing the haemoglobin to pass through the filter into a filtrate channel which was then discarded. The membranes were pumped back into a reservoir to which lysis buffer was added and the membranes were then allowed to flow back over the filter where more haemoglobin was removed and the retained membranes were cycled back to the reservoir to repeat the process until the membranes were completely free of haemoglobin. Erythrocyte membranes were washed in 100 litres of lysis buffer to remove all haemoglobin. In the final cycle 2.2 litres of solubilisation buffer were added to the membranes in the reservoir. This was collected and the E were left to solubilise overnight at 4°C. The insoluble membrane fragments were separated from the supernatant by centrifugation at 50,000g for 30 min at 4°C. The supernatant containing solubilised CR1 was diluted with 500mls of ice-cold water to lower the conductivity to 6mS at 0°C and applied to a Biorex column (5x30cm). This column was eluted with a linear sodium chloride gradient as before. Thereafter a similar chromatographic procedure to that described for the first and second purification attempts was followed with some modifications which will be described in the results section. All columns in the third purification attempt were screened by measuring the ability of each fraction to agglutinate EAC43b.

Results

## 2.12 First CR1 Purification Attempt

The starting material for the Biorex column contained approximately 750mg of protein which represented 47% of the original solubilised membranes (Table 2.1). This was eluted from the Biorex by application of a linear sodium chloride gradient. The conductivity and protein concentration of each fraction was recorded and the column was then screened for CR1 activity by radioimmunoassay (Fig 2.6). Two peaks of CR1 activity eluted between 8 and 13mS and the fractions containing this activity were pooled. The pool contained approximately 171mg of protein representing 23% of the applied protein. After concentration using an Amicon PM-30 membrane and dilution with water to lower the conductivity the material was applied to a C3-Sepharose column. Elution of this column revealed 2 fractions which contained CR1 activity (Fig 2.7). As the exclusion fractions also contained CR1 activity these were concentrated as before and recycled over the C3-Sepharose column. The fractions containing CR1 activity which eluted from this second column were added to the pool from the first column and the concentrated pool which contained 15.3mg of protein, representing 15% of the applied protein, was subjected to gel filtration on a Biogel column.

CR1 did not filter as a distinct peak from this column but represented a broad band of activity between 50% and 80% of the bed volume (Fig 2.8). The pool of CR1 activity from the Biogel was concentrated to 11mls using an Amicon PM-10 membrane. This contained 2.42mg of protein representing 6.8% of the applied protein. The pool from the Biogel column was subjected to affinity chromatography on a 1ml lentil lectin column and the bound CR1 eluted with equilibrating buffer containing α methylmannoside (0.2 mol $l^{-1}$). Each fraction was dialysed into VBS 0.1% NP-40 and assayed for CR1 activity (Fig 2.9). No CR1 activity appeared to have eluted from this column and so a higher concentration (0.5 mol $l^{-1}$ and 1 mol $l^{-1}$) of α methylmannoside was added to the equilibrating buffer in an effort to elute any protein which may have stuck to the column. This again proved unsuccessful.

To exclude the possibility that CR1 was present, but was not being detected fractions were screened for their ability to decay dissociate C3bBbP as CR1 is known to accelerate the decay of this enzyme (Fearon 1979, Iida and Nussenzweig 1981). However, none of the fractions contained convertase activity. Thus it appeared that all CR1 activity had been lost.

## 2.13 Second Attempt at CR1 Purification

A second CR1 purification was attempted using 15 units of packed red cells with a protein content of 3600mg. The same protocol for obtaining the solubilised receptor was used and the resultant material containing 2680mg of protein was applied to a Biorex column. This gave a similar

Table 2.1 Protein Profile for the First CR1 Purification

| | Total Protein | CR1cpm(25µl) | Total cpm | cpm/mg | Volume |
|---|---|---|---|---|---|
| Solubilised Membranes | 1600mg | ND | ND | ND | 1500ml |
| Solubilised Supernatant | 750mg | 168 | $10x10^6$ | 13333 | 1500ml |
| Pool from Biorex | 171mg | 736 | $3,6x10^6$ | 21052 | 125ml |
| Pool from C3-Sepharose | 15,3mg | ND | ND | ND | 5ml |
| Pool from Biogel | 2,42mg | 246 | $0,11x10^6$ | 44628 | 11ml |
| Pool from Lentil lectin | 0 | 0 | 0 | 0 | |

Fig 2.6 Biorex Column

Legend

Purification of CR1 from solubilised E membranes by passage over a Biorex column (5x30cm). Fractions (10ml) were collected and screened for CR1 activity by RIA (x_____x). Protein concentration was determined by Folin analysis (x_._._.x) and conductivity was measured at 0°C (x----x).The arrows on the X axis represent: 1 the application of the solubilised membranes to the column, 2 the washing of the column with equilibrating buffer and 3 the application of a linear sodium chloride gradient. Fractions 245 to 285 were pooled as they contained the greatest CR1 activity.

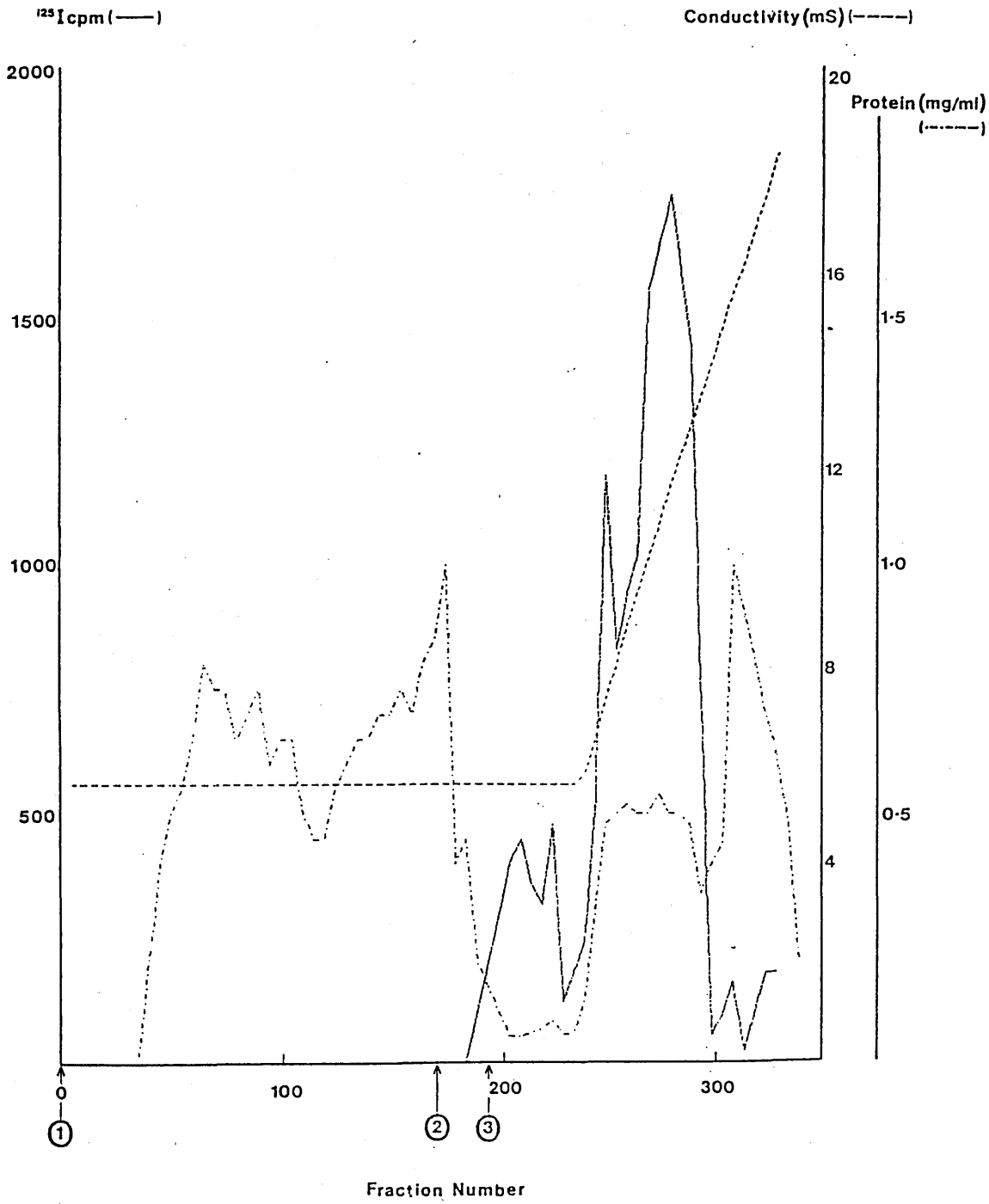

$^{125}$I cpm (——)
Conductivity (mS) (– – – –)
Protein (mg/ml) (–·–·–·–)
2000
1500
1000
500
20
16
12
8
4
1·5
1·0
0·5
0
100
200
300
①
②
③
Fraction Number

Fig 2.7 C3-Sepharose Column

Legend

Purification of CR1 from the concentrated pool obtained from the Biorex column by passage over a C3-Sepharose column (10ml). Fractions (10ml) were collected and screened for CR1 activity by RIA (x----x). Protein concentration was determined by Folin analysis (x_____x). The arrows on the X axis represent: 1, application of the Biorex pool, 2, wash with equilibrating buffer, 3, wash with equilibrating buffer containing 0.1% NP-40, 4, wash with equilibrating buffer containing sodium chloride (0.2 mol $l^{-1}$), 5, application of elution buffer containing NP-40 and sodium chloride. Fractions 47 and 48 were pooled as well as fractions 5 to 11 from the exclusion peak.

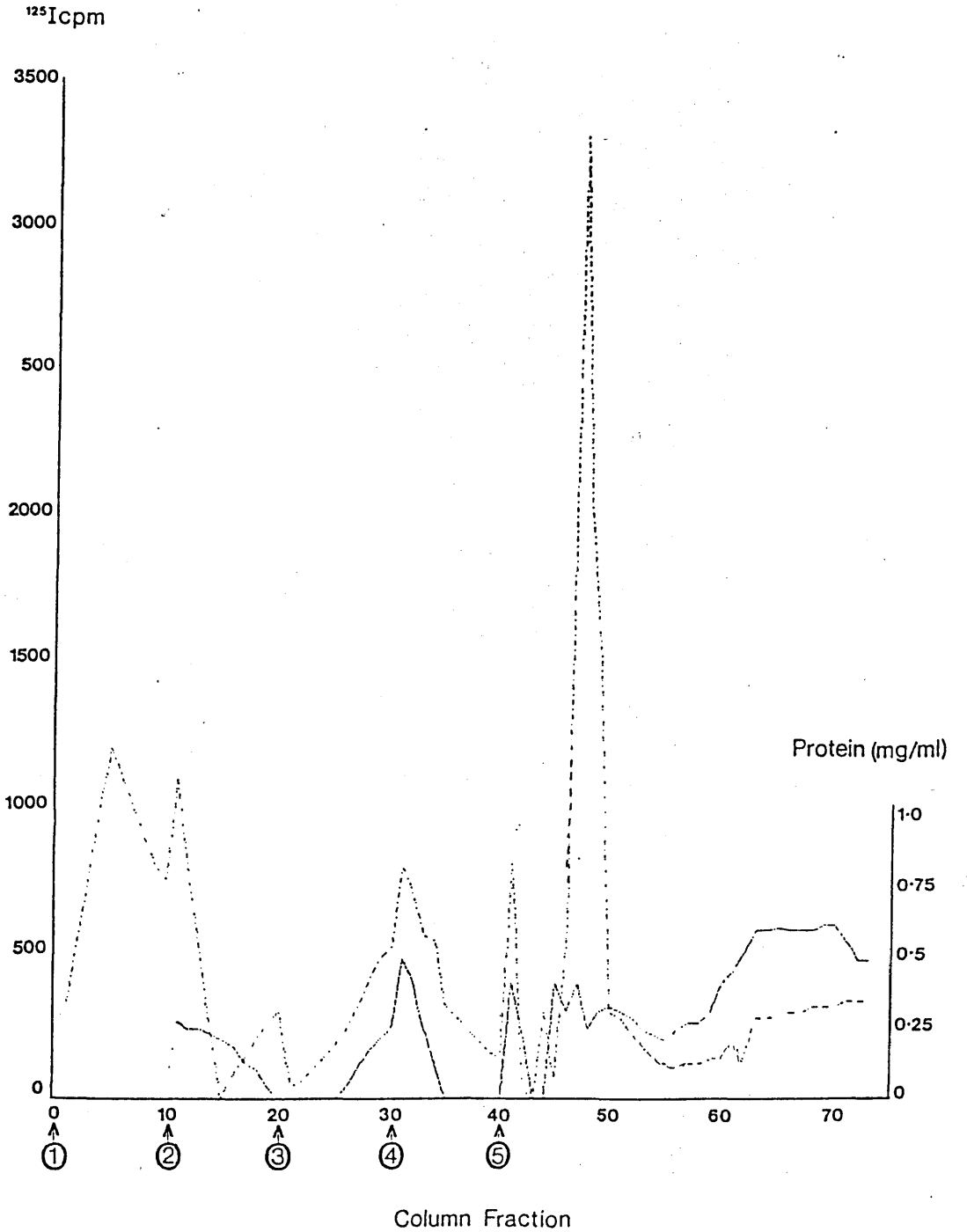

$^{125}$Icpm
3500
3000
500
2000
1500
1000
500
0
Protein (mg/ml)
1·0
0·75
0·5
0·25
0
0
10
20
30
40
50
60
70
①
②
③
④
⑤
Column Fraction

Fig 2.8 Biogel Column

Legend

Purification of CR1 from the concentrated pool obtained from the C3-Sepharose column by application to a Biogel gel filtration column (2.6x90cm). Fractions (3ml) were collected and screened for CR1 activity by RIA (x_____x). Protein concentration was determined by Folin analysis (x_._._.x). Fractions 47 to 63 were pooled.

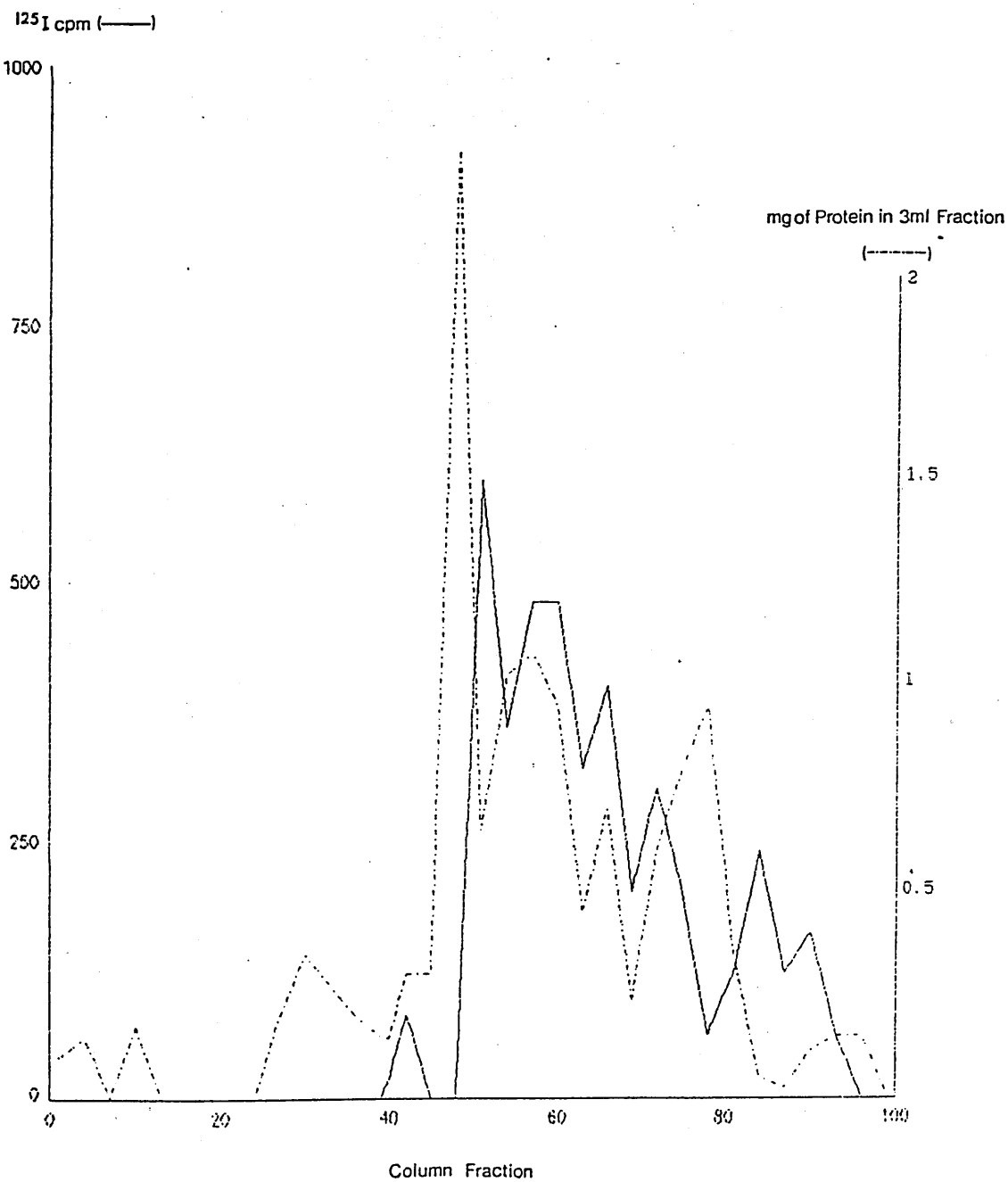
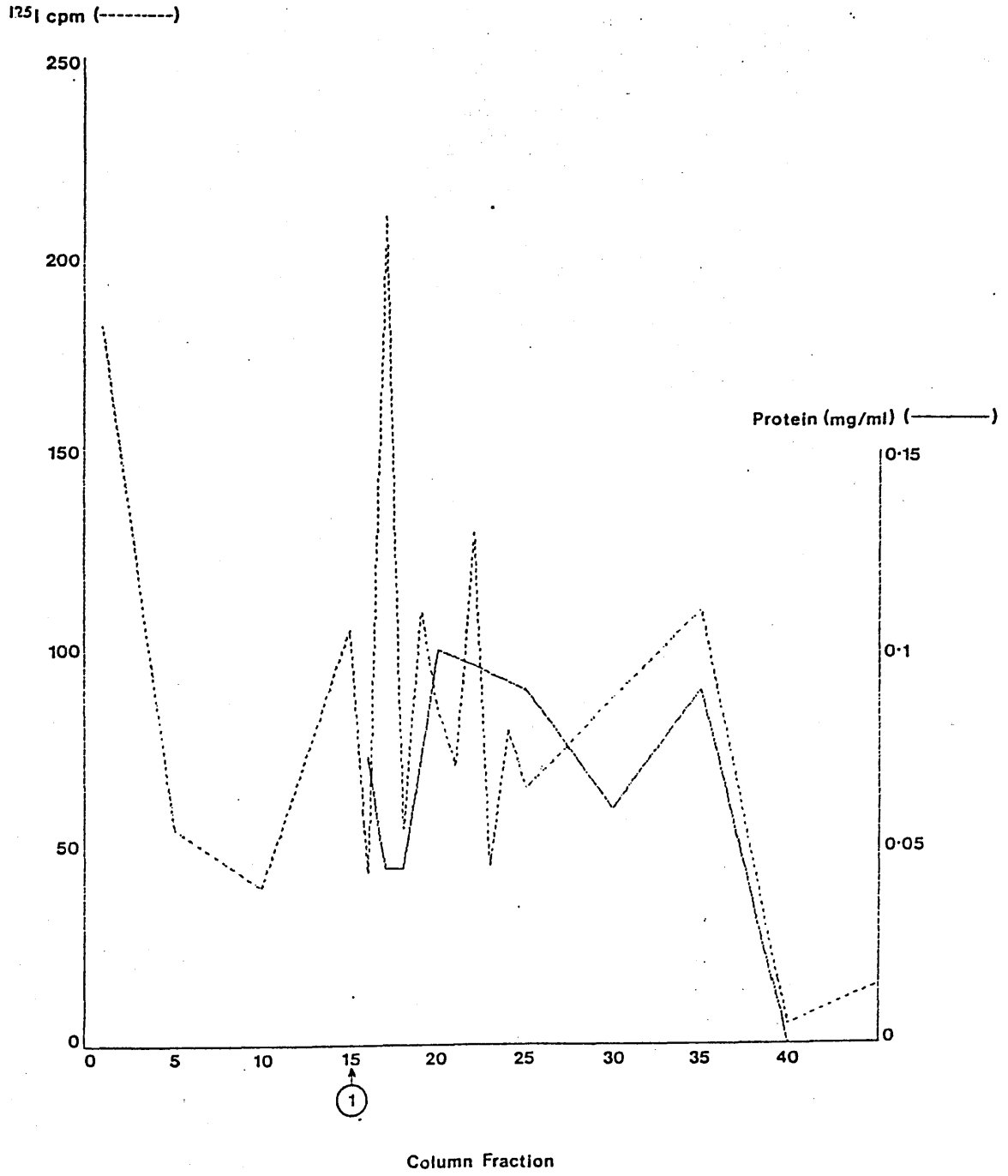

$^{125}$I cpm (——)
1000
750
500
250
0
mg of Protein in 3ml Fraction
(- - - - -)
2
1.5
1
0.5
0
20
40
60
80
100
Column Fraction

Fig 2.9 Lentil Lectin Column

Legend

Purification of CR1 from the concentrated Biogel pool by application to a 1 ml lentil lectin affinity chromatography column. Fractions (1ml) were collected and screened for CR1 activity by RIA (x----x). Protein concentration was determined by Folin analysis (x_____x). The arrow (1) on the X axis represents the application of the elution buffer.


$^{125}$I cpm (---------)
250
200
150
100
50
0
Protein (mg/ml) (————)
0·15
0·1
0·05
0
0
5
10
15
20
25
30
35
40
1
Column Fraction

profile to the one shown in Fig 2.6 with CR1 activity eluting between 8 and 13mS. The CR1 active fractions from this column were concentrated down to a pool of 80mls containing 142mg of protein. This pool was divided into 4 lots of 20mls. Each 20mls was then diluted with 100mls of ice-cold water prior to application to a C3-Sepharose column. All of the CR1 active fractions from each column were pooled to give a pool of 48mls with a total protein content of 2.12mg. Instead of putting this material over a Biogel column, the pool was dialysed into the equilibrating buffer for the Biorex column and put over a 1ml Biorex column to concentrate CR1 activity. Elution of this column with equilibrating buffer containing sodium chloride (0.6 mol $l^{-1}$) resulted in no detectable CR1 activity in any fraction as assessed by RIA or the C3bBbP convertase inhibition assay.

## 2.14 Purification of CR1 Using the Pellicon Cassette System

The membranes from 18 units of mixed fresh and expired blood were collected and washed until they were free of haemoglobin in a Pellicon cassette. This procedure took 1 day. The haemoglobin free membranes were then solubilised as before, centrifuged and the supernatant containing the solubilised CR1 retained. The protein content of this starting material was 2015mg, it was diluted with 500mls of ice-cold water and applied to a Biorex column which was washed and eluted as previously described. Each fraction was screened for CR1 activity by agglutination of EAC43b (Fig 2.10).

CR1 activity eluted as a single peak between 8 and 10mS. The fractions containing the highest CR1 activity were pooled and concentrated to 234mls. This pool gave a positive haemagglutination at up to a 1 in 320 dilution (Plate 2.1), and contained 166mg of protein (Table 2.2) representing 8.2% of the starting material. One hundred millilitres of this pool were diluted with 400mls of ice-cold water and the remainder was stored at -70°C. The diluted material was applied to C3-Sepharose and eluted as before (Fig 2.11). The protein content of the material eluted from this column could not be accurately measured, however, positive haemagglutination was seen in a number of the fractions, with the highest titre being 1:1280. Fractions containing CR1 activity were pooled and dialysed into the equilibrating buffer for the lentil lectin column. This pool which had a total volume of 45mls with agglutinating activity of 1:320 was applied to a lentil lectin column. Elution with α-methylmannoside(0.2 mol $l^{-1}$) revealed that CR1 activity eluted as 2 very sharp peaks with peak CR1 activity at 1:1280 dilution (Fig 2.12) (Plate 2.1). Two pools were made from these fractions. The first pool was concentrated to 2mls and contained 48μg/ml of protein while the second was concentrated to 2.5mls and contained 32μg/ml of protein. Samples from both of these pools were analysed by SDS-PAGE on 5% gels. The gels were either silver stained or stained with coomassie brilliant blue. Silver stain analysis of both pools revealed a band of molecular weight 230,000 daltons (Plate 2.2). No minor lower molecular weight contaminating bands were present indicating that the preparation was homogeneous. Samples of

Fig 2.10 Biorex Column

Legend

Purification of CR1 from solubilised E membranes by passage over a Biorex column (5x30cm). Fractions 15ml were collected and screened for CR1 activity by agglutination of EAC43b (x_____x). Protein concentration was measured by Folin analysis (x_._._.x) and conductivity was measured at 0°C (x-----x). The arrows on the X axis represent: 1, application of protein, 2, wash with equilibrating buffer and 3, application of a linear sodium chloride gradient. Fractions 270 to 300 were pooled as these contained the greatest CR1 activity.

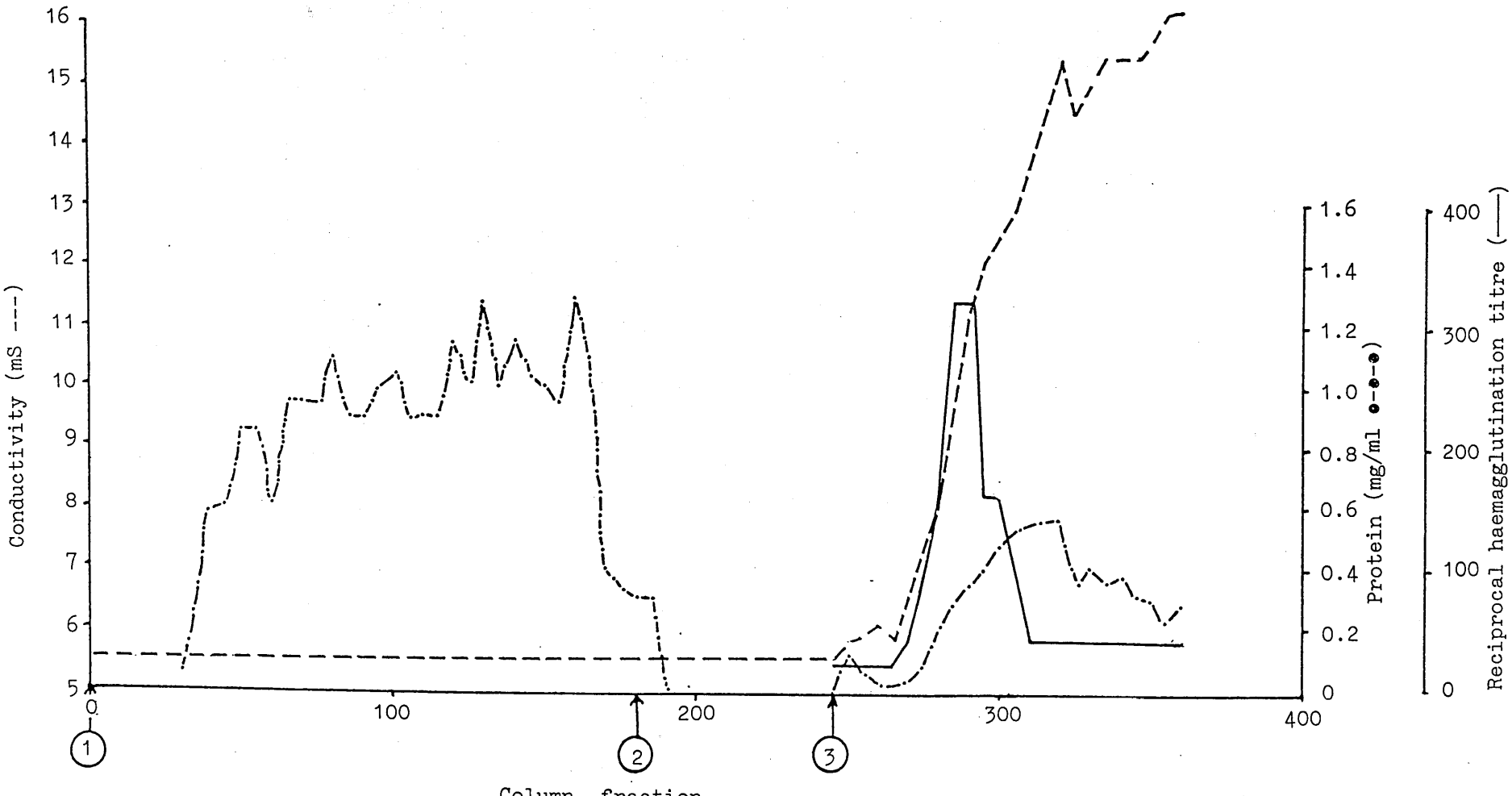

Conductivity (mS ---)
16
15
14
13
12
11
10
9
8
7
6
5
0
100
200
300
400
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Protein (mg/ml •-•-•)
400
300
200
100
0
Reciprocal haemagglutination titre (——)
1
2
3
Column fraction

Plate 2.1 Haemagglutination of EAC43b

A sample (5µl) of fraction 27 from the lentil lectin column (Fig 2.12) was serially diluted out in PBS to 1:2500 (row 1). A sample (5µl) of fraction 285 from the Biorex column (Fig 2.10) was also serially diluted out in PBS to 1:2500 (row 2), as was a sample (5µl) of an exclusion fraction from the Biorex column (Fig 2.10) (row 3). After addition of EAC43b the haemagglutination pattern was noted. Row 1 shows the haemagglutination at up to a 1:1280 dilution of purified CR1 from the lentil lectin column, row 2 shows the haemagglutination of EAC43b at up to a 1:320 dilution of CR1 from the Biorex pool and row 3 shows the haemagglutination of EAC43b at up to a 1:20 dilution of the exclusion fraction from the Biorex column. The last well in each row represents the control wells in which no test sample was added.

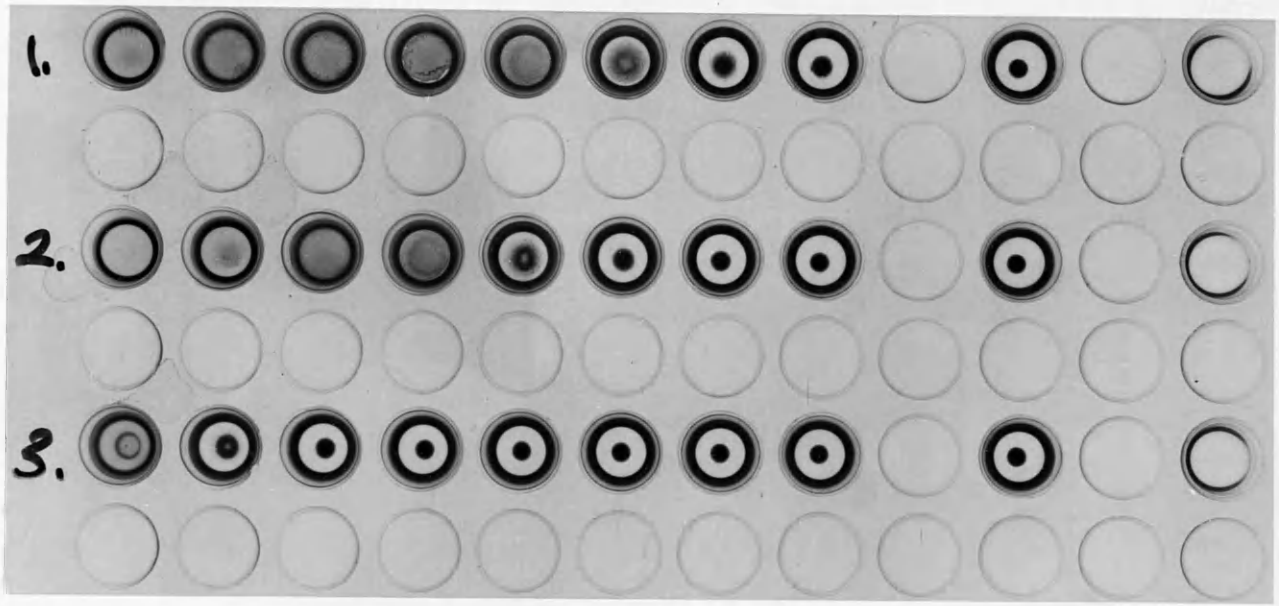

1.
2.
3.

Table 2.2 Protein Profile for the Third CR1 Purification

| | Protein(mg/ml) | Haemagglutination Titre | Volume |
|---|---|---|---|
| Solubilised Membranes | ND | 1:160 | 1300ml |
| Solubilised Supernatant | 1.55 | 1:160 | 1300ml |
| Pool from Biorex | 0.71 | 1:320 | 234ml |
| Pool from C3-Sepharose | 0* | 1:320 | 45ml |
| Pool from Lentil Lectin | (i)48µg/ml<br>(ii)32µg/ml | 1:1280<br>1:1280 | 2ml<br>2.5ml |

Note: 0*; No protein could be detected.

Fig 2.11 C3-Sepharose Column

Legend

Purification of CR1 from the concentrated pool obtained from the Biorex column by application to a C3-Sepharose affinity chromatography column (10ml). The arrows on the X axis represent: 1, application of protein (15ml exclusion fractions were collected), 2, wash with equilibrating buffer (5ml fractions were collected), 3, wash with equilibrating buffer containing NP-40 (5ml fractions were collected), 4, wash with equilibrating buffer (5ml fractions were collected), 5, wash with equilibrating buffer containing sodium chloride (0.2 mol $l^{-1}$) (5ml fractions were collected), 6, wash with equilibrating buffer (5ml fractions were collected) and finally, 7, application of elution buffer (2ml fractions were collected). Fractions were screened for CR1 activity by agglutination of EAC43b (x_____x) and protein concentration was determined by Folin analysis (x_._._.x). Fractions 92 to 104 were pooled as these contained the greatest CR1 activity.

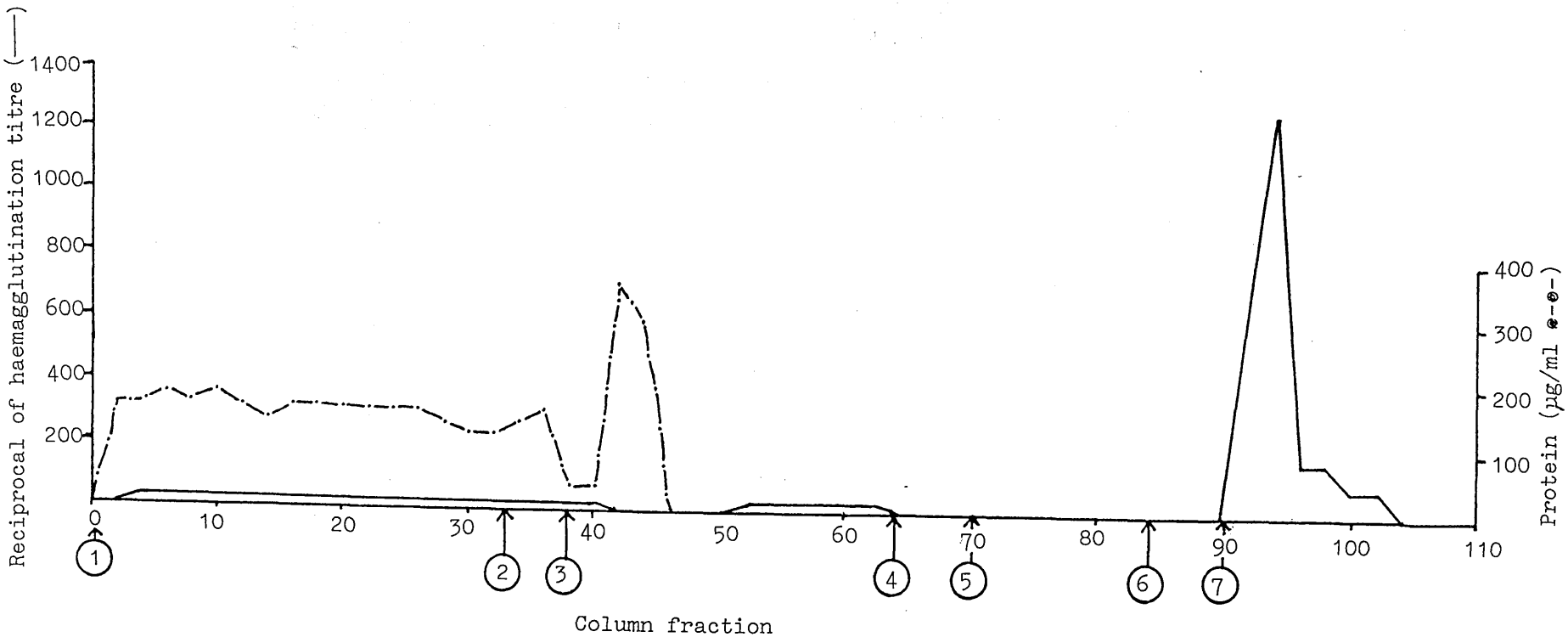

Reciprocal of haemagglutination titre (——)
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
60
70
80
90
100
110
400
300
200
100
Protein (µg/ml ●-●-)
1
2
3
4
5
6
7
Column fraction

Fig 2.12 Lentil Lectin Column

Legend

Purification of CR1 from the concentrated C3-Sepharose pool by application to a 1ml lentil lectin affinity chromatography column. Fractions (2ml) were collected and screened for CR1 activity by agglutination of EAC43b (x_____x). No protein could be detected by Folin analysis. The arrows on the X axis represent: 1, application of protein, 2, wash with equilibrating buffer and 3, application of elution buffer. Two pools were made, the first containing fractions 23 to 25 and the second containing fractions 26 to 31.

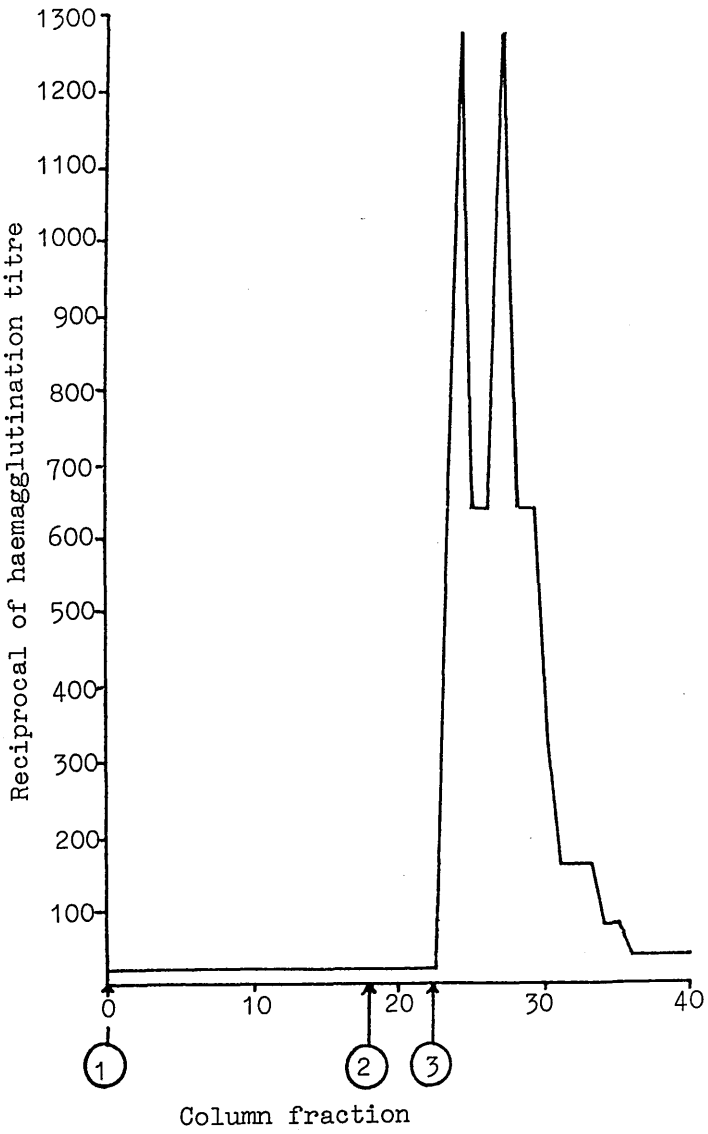
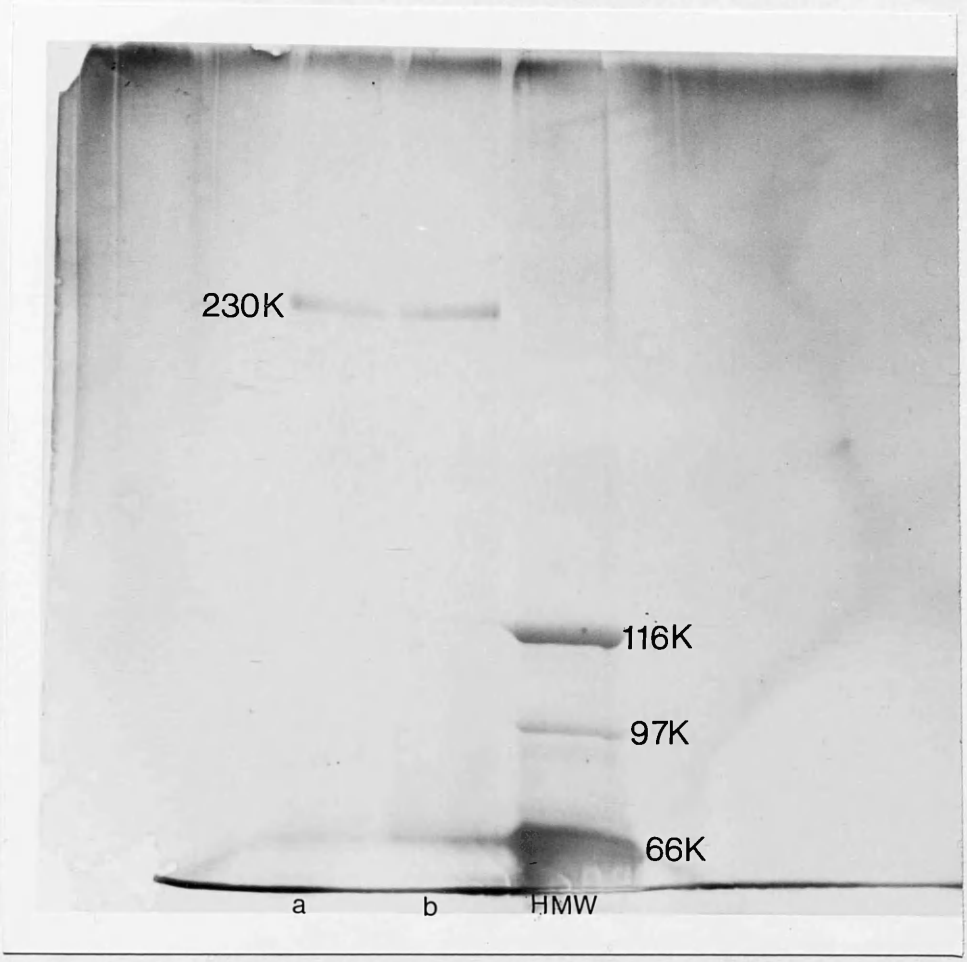

1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
Reciprocal of haemagglutination titre
0
10
20
30
40
1
2
3
Column fraction

Plate 2.2 Silver Stain of Purified CR1

The purity of the CR1 which was obtained from the lentil lectin column was assessed by SDS-PAGE analysis. Samples from both of the pools from the lentil lectin column (Fig 2.12) were analysed on a 5% slab gel run under reducing conditions. A band with a molecular weight of 230,000 daltons (230K) was seen in both cases (track a, pool 1; track b, pool 2) after the gel was silver stained. No other contaminating bands were observed. The last track represents the high molecular weight markers (HMW).


230K
116K
97K
66K
a
b
HMW

the lentil lectin beads were also analysed by SDS-PAGE and silver staining revealed a band with a molecular weight of 230,000 daltons (Plate 2.3)

## 2.15 Specificity of the CR1 Antiserum

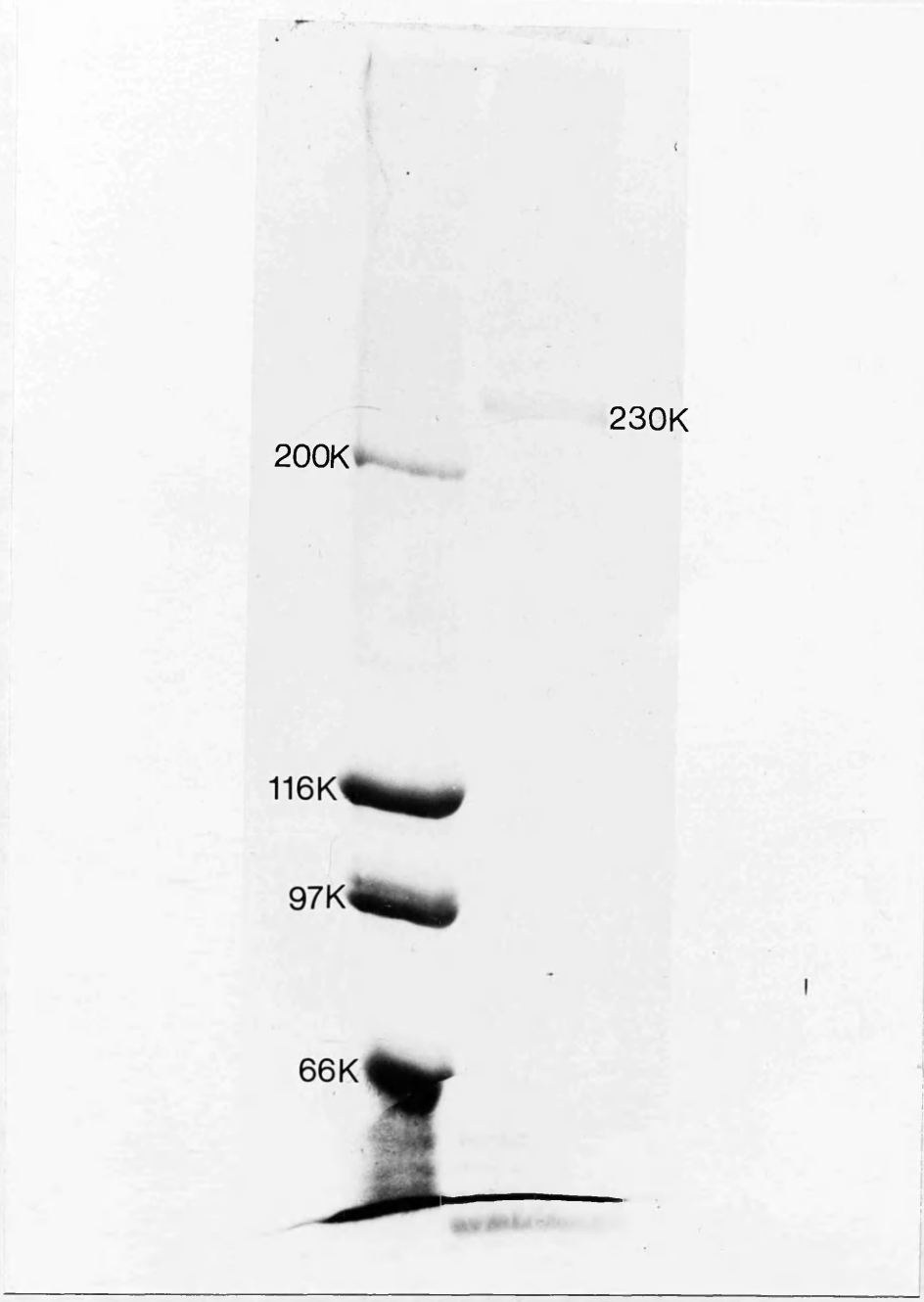
Antiserum to the purified CR1 was raised in rabbits. The serum obtained from 20mls of clotted rabbit blood was serially diluted into PBS and tested against purified CR1 by double immunodiffusion in agarose gel. Single lines of precipitation were seen between CR1 and each of the diluted anti-CR1 samples. This indicated that the antiserum was specific for CR1 (Plate 2.4).

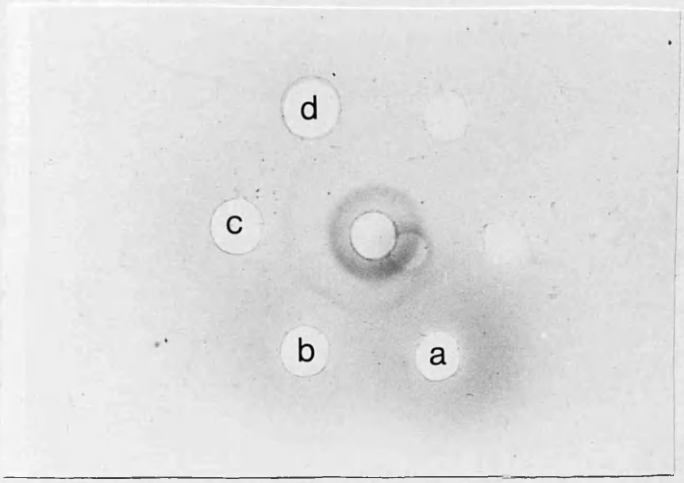
Plate 2.3 Silver Stain of Sepharose Beads from the Lentil Lectin Column

To determine if CR1 had stuck irreversibly to the lentil lectin column samples of Sepharose beads from the column (Fig 2.12) were analysed on a 5% slab gel run under reducing conditions. A band of molecular weight 230,000 daltons was seen after silver staining (track a). This is identical to the molecular weight of purified CR1 and it was therefore concluded that this band represented CR1. The first track represents the high molecular weight markers (HMW).

Plate 2.4 Immunodiffusion in Agarose Gels Between Anti-CR1 and CR1

Double immunodiffusion in agarose gels of purified CR1(48μg/ml), (central well) against rabbit anti-CR1 antiserum, 1mg/ml (well a), 0.5mg/ml (well b), 0.25mg/ml (well c) and 0.125mg/ml (well d). A precipitin line of identity was formed between CR1 and each of the anti-CR1 samples.


230K
200K
116K
97K
66K


d
c
b
a

## 2.16 Discussion

It was realised after the failure of the first 2 preparations that, as CR1 was an extremely labile protein (Sim 1985; Ripoche and Sim 1986), some modifications would have to be made to the purification procedure. It was decided to shorten the length of the procedure in 2 ways, (i) by speeding up the washing of the E membranes and (ii) by using a quicker method of screening the columns. In addition extra protease inhibitors were added to the buffers in an attempt to cut down proteolysis of the receptor.

In order to decrease the time spent washing the membranes, a Pellicon cassette system was used. This system had previously been successfully utilised by Wong et al (1985) in their preparation of CR1. The efficiency with which this machine operated meant that the cells were ready to be put over the Biorex column within 24 hours of beginning the preparation. As both the RIA and the C3bBbP convertase inhibition assay were time consuming, agglutination of EAC43b was used instead to screen the columns. This was a sufficiently reliable method to ensure that CR1 activity could be detected quickly and accurately.

Two modifications were made to the chromatographic procedures, (i) only 100mls of the concentrated pool from the Biorex column were applied to the C3-Sepharose column. This helped to decrease the overloading of the column which had occured in the first preparation (Fig 2.7) and (ii) the Biogel column was not used as previous experience had suggested that this column did not greatly add to the purity of CR1 and may have in fact diluted the receptor pool (Fig 2.8). The pool from the C3-Sepharose column was therefore dialysed into the equilibrating buffer for the lentil lectin column, followed by application to the column and successful elution of 2 peaks of CR1 activity by $\alpha$ methylmannoside (0.2 mol $l^{-1}$) (Fig 2.12). When pooled these 2 peaks both gave a positive haemagglutination titre of 1:1280. The first of these pools when concentrated had a total protein content of 96$\mu$g and the second a total of 80$\mu$g. This gave a combined total of 176$\mu$g of purified CR1 from 100mls of the original Biorex pool. If the remaining 134mls from the Biorex pool were to yield a comparable amount of CR1 then it can be estimated that from this purification 412$\mu$g of CR1 were obtained. This is 3 times in excess of the amount of purified CR1 which Fearon obtained although the amount of protein in the initial Biorex starting material was comparable in both cases (Fearon 1979).

The purified CR1 was analysed by 5% SDS-PAGE and a single band of molecular weight 230,000 daltons in the reduced form was seen with coomassie blue staining. When the gel was silver stained, a process which is 2- to 5-fold more sensitive than coomassie blue staining, no additional bands were observed indicating that the preparation was pure (Plate 2.2).

The specificity of the anti-CR1 antiserum was shown by the observation that a single precipitin line was formed when it was tested by double diffusion in agarose gels against purified CR1 (Plate 2.4).

# CHAPTER THREE

## 3.1 Introduction

In 1965 Klopstock et al (1965) observed a wide variation in the immune adherence activity of E from different individuals. From the results of family studies they concluded that the capacity of E to mediate immune adherence was inherited. Since that time it has become generally accepted that E CR1 numbers in the normal population are inherited (Wilson et al 1982; Minota et al 1984; Walport et al 1985a; Holme et al 1986). Wilson et al (1982) enumerated CR1 on E from a section of the normal population and found that the numbers appeared to fall into a trimodal distribution. On this basis they suggested the existence of a genetic locus with 2 codominant alleles (encoding high (H) and low (L) expression) determining the level of E CR1 expression. The data of Walport et al (1985a) also indicated that in the normal population there was a heritable component in the expression of CR1 number. However this group did not agree with the simple model of inheritance proposed by Wilson et al (1982) considering it more likely to be under polygenetic control.

The numerical polymorphism of CR1 has aroused considerable interest due to the discovery that patients with SLE have reduced E CR1 levels. This finding has been unequivically proven by studies in laboratories from different parts of the world (Miyakawa et al 1981; Iida et al 1982; Wilson et al 1982; Taylor et al 1983a; Ross et al 1985; Holme et al 1986). The mechanism whereby this reduction occurs is unknown, although 2 general mechanisms have been proposed, (i) low CR1 numbers are inherited by means of an allele(s) associated with low CR1 number or (ii) they are acquired as a result of a pathological process. Most recent evidence indicates that low E CR1 numbers in SLE patients are acquired with the level of CR1 expression being related to disease activity (Ross et al 1985; Holme et al 1986).

In order to compare the influence of genetic and environmental factors on E CR1 expression in the normal population, it was decided to undertake a study of E CR1 levels in twins. The twin study method was first proposed as a technique for differentiating between the influence of heredity and environmental factors by Sir Francis Galton (1875). He reasoned that since monozygotic twins have exactly the same hereditary structure, any difference between the pair must be caused by environmental factors. The converse, that if a pair of monozygotic twins are alike in any given trait that this trait is inherited is not necessarily true as twins usually share a close common environment. Unless this limitation is recognised the role of genetic factors may be overestimated. Thus the twins study method is possibly more useful for demonstrating that environmental rather than hereditary factors are important in the development of any given trait (Buchanan et al 1967).

In addition to measuring E CR1 numbers on the twins, the serum levels of C4bp, H and I were measured by enzyme linked immunosorbent assay (ELISA). CR1 is known to be closely related in function and binding activity to the other cofactor proteins H and C4bp (Iida and Nussenzweig

1981). Moreover Rodriguez de Cordoba et al (1985) have shown that the structural genes for CR1, H and C4bp are closely linked and recently the structural genes for CR1, H and C4bp have all been mapped to chromosome 1 (Klickstein et al 1985). The genetic linkage and the functional homologies have led to the hypothesis that these 3 proteins, (and possibly DAF and gp45-70 also) may comprise a new human complement-regulatory gene family analogous to the C4/C2/B and C6/C7 gene families already described (Holers et al 1985).

This chapter will describe the analysis which was performed to determine the relative roles of genetic and environmental factors on E CR1 expression and on the serum concentrations of H, C4bp and I. In addition the levels of each of the proteins were examined to see if age or sex had any influence on them. Finally an analysis was carried out to determine if there were any correlations evident between CR1, C4bp, H and I.

Materials and Methods

## 3.2 Chemicals and Reagents

Chemicals and other reagents were obtained from the following sources:

Sigma Chemical Company, Fancy Rd, Poole, Dorset.

Caprylic Acid
Hydrogen Peroxide ($H_2O_2$)
o-Phenylene-Diamine (OPD)
Polyvinyl Pyrrolidone (PVP)

Dynatech Laboratories Ltd, Daux Rd, Billingshurst, Sussex.

Flat Bottomed Micro-Elisa Plates

Flow Laboratories, Irvine, Ayrshire

RPMI-1640 medium without sodium bicarbonate, without glutamine, with HEPES.

Pharmacia House, Midsummer Boulevard, Milton Keynes

Sephadex G-50, (medium)

Pierce and Warriner UK Ltd, 44, Upper Northgate St, Chester, Cheshire

Iodo-Beads

British Oxygen

Liquid nitrogen was supplied by British Oxygen.

## 3.3 Twins

One hundred and twenty three sets of twins were recruited into this study as a result of an advertising campaign on radio and in the local and national press. The zygosity, sex and age distribution of the twins are shown in Fig 3.1. Both members of each twin pair were interviewed at the same time and the presence of existent disease noted. None of the twins had any chronic inflammatory rheumatic disease.

## 3.4 Blood Samples

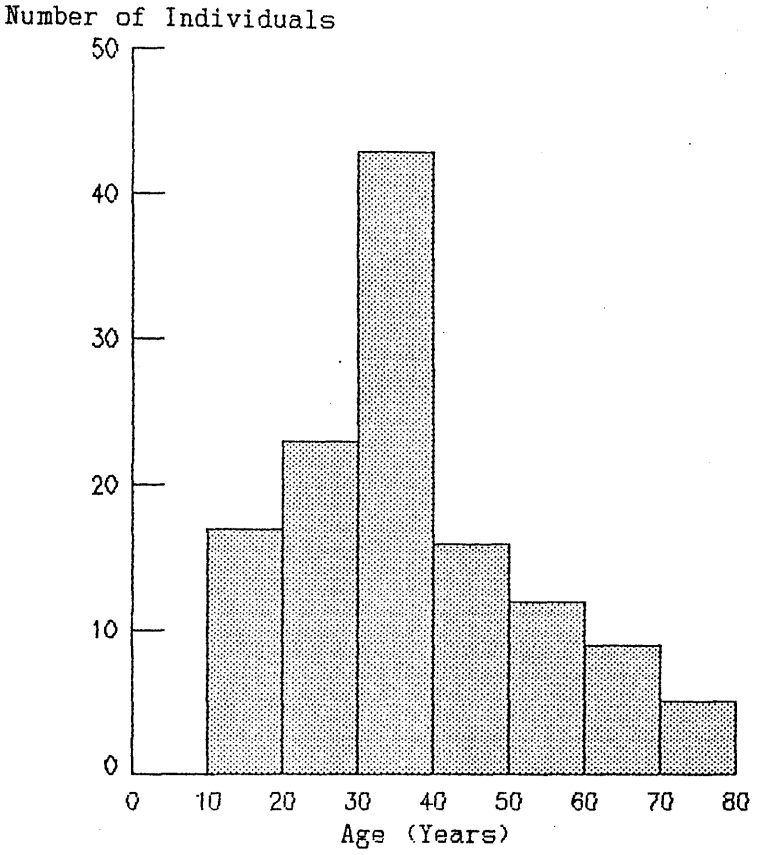
At the time of interview, 60mls of venous blood were taken, 40mls were allowed to clot at room temperature for 1 hour and then incubated on ice for 30 min to allow the clot to retract. The clot was separated from the

Fig 3.1 Zygosity, Sex and Age Distribution of the Twins

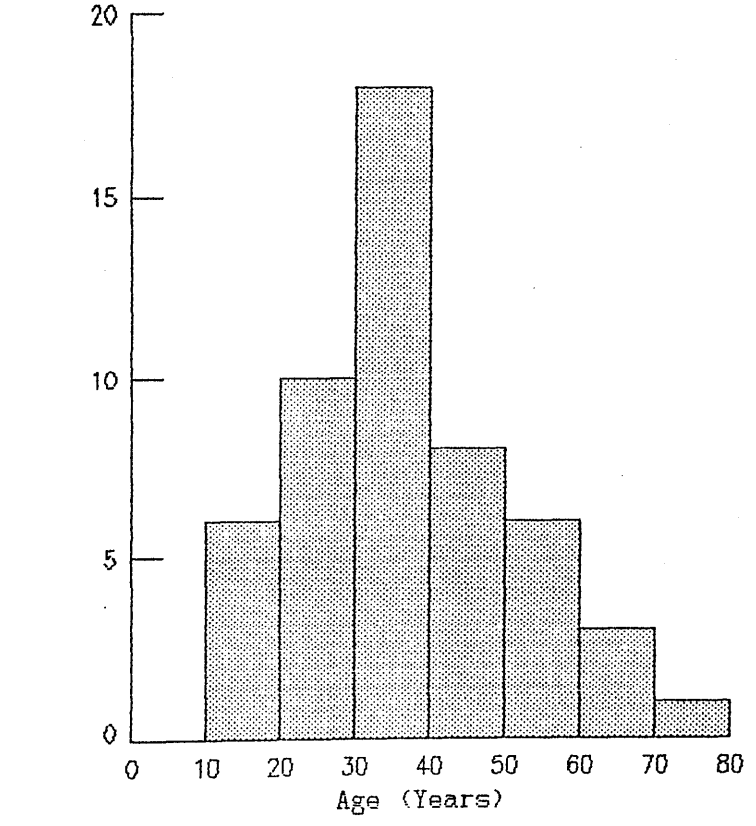
Legend

Frequency histograms showing the age distribution of each set of twins; (a) all twins, (b) monozygotic female, (c) monozygotic male, (d) dizygotic female, (e) dizygotic male, (f) dizygotic male and female.

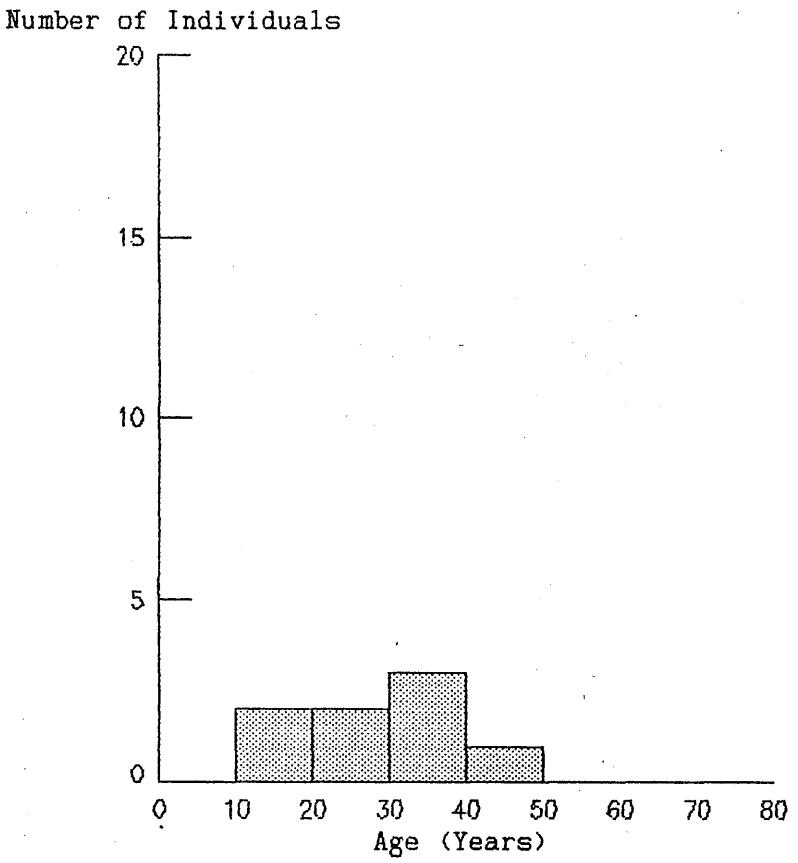
(a)


Number of Individuals
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Age (Years)

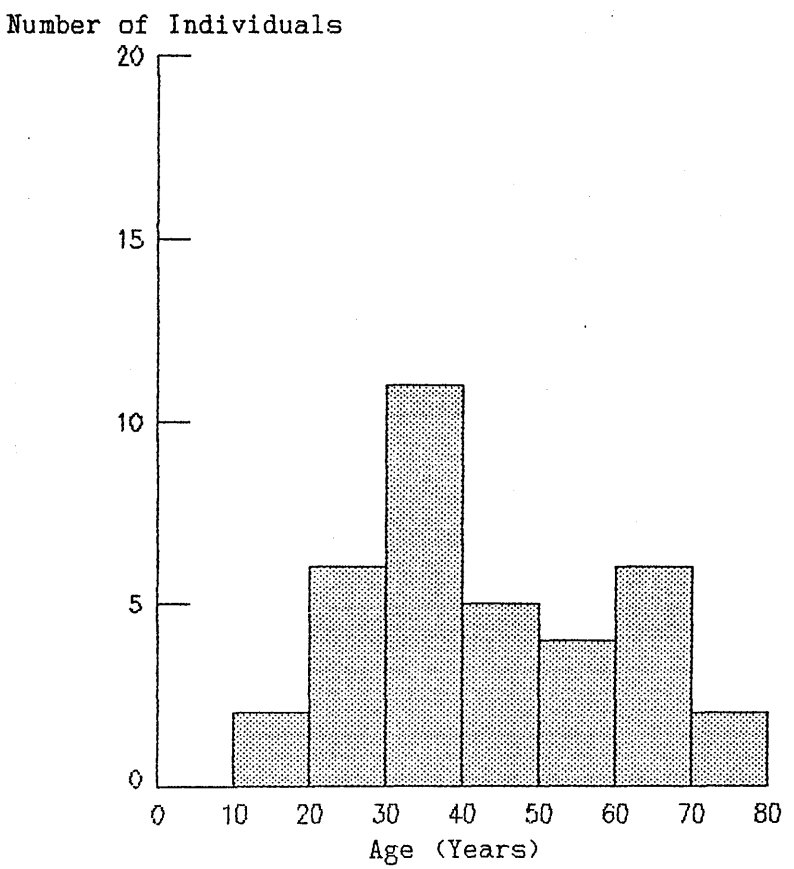
(b)


Number of Individuals
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Age (Years)

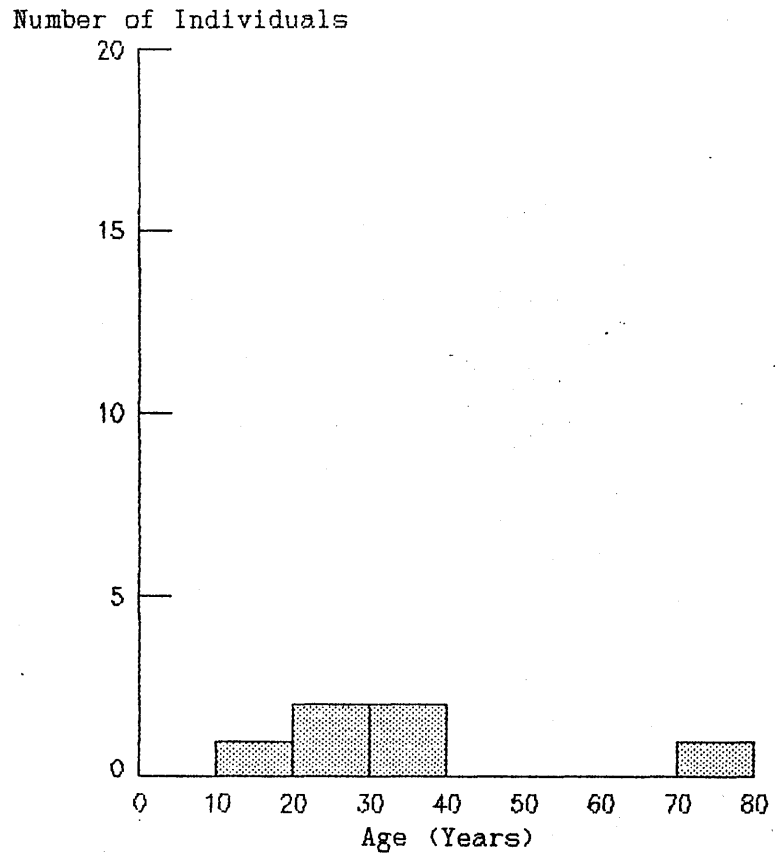
(c)


Number of Individuals
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Age (Years)

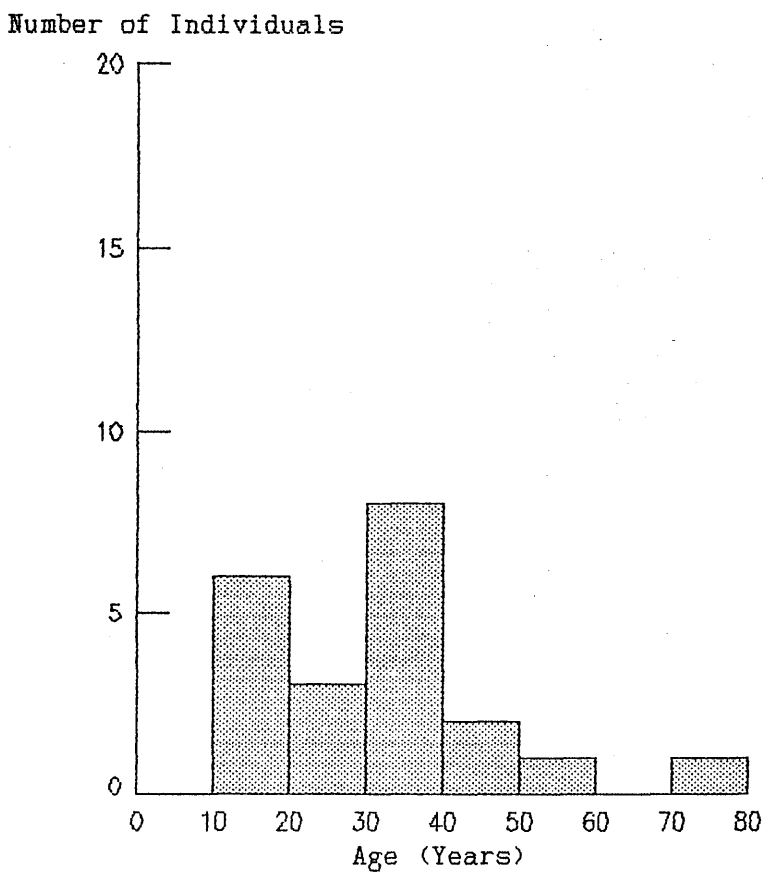
(d)


Number of Individuals
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Age (Years)

(e)


Number of Individuals
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Age (Years)

(f)


Number of Individuals
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Age (Years)

serum by centrifugation at 2000g for 5 min at room temperature. The serum was removed and stored in aliquots at -70°C.

The remaining 20mls of blood were anticoagulated with heparin and frozen as described in section 3.5.

3.5 Freezing Blood Samples

Reagents

30%(w/v) Polyvinyl/Pyrrolidone (PVP)

30g of polyvinyl/pyrrolidone were added to 100mls of PBS in a glass bottle. The bottle was then put into a pressure cooker and heated until the polyvinyl/pyrrolidone had dissolved.

RPMI/BSA

1g of BSA was added to 100mls of RPMI.

Procedure

Six millilitres of 30%(w/v) PVP were added dropwise to 20mls of heparinised blood from each of the twins. Using a Pasteur pipette the blood/PVP mixture was then added dropwise into a steel bowl containing liquid nitrogen. The individual frozen pellets were collected and stored at -70°C in plastic universals prior to measurement of CR1 numbers and blood group analysis.

3.6 Thawing Blood Samples

When required the blood droplets were thawed out by adding one droplet to 0.5mls of warm (37°C) RPMI. The thawed cells were centrifuged at 2000g for 5 min at room temperature and the buffy coat removed by aspiration. As the thawing process resulted in a variable degree of lysis each twin sample was given 5 50ml washes in warm (37°C) RPMI and resuspended in RPMI/BSA. One hundred microlitres of the E suspension was then added to 2.9mls of deionised water and the optical density was measured at 541nm. The E were adjusted to $2.5 \times 10^8$/ml using the following formula:

$$\text{Volume of E required} = \text{Initial volume} \times \frac{OD_{541} \text{ Measured}}{0.185} \times 2$$

3.7 Determination of Zygosity

The first priority in any twins study is to establish the zygosity of the twins. In this case zygosity was determined by similarities in the physical appearance of the twins (eg. hair colour, eye colour, facial appearance) and the reply to the question, "Have you always been

considered to be identical twins?". On this basis 61 pairs were considered to be monozygotic and 62 pairs dizygotic. Erythrocytes from the monozygotic twins were then subjected to blood group analysis. This analysis was carried out by the Department of Haematology in the Western Infirmary, Glasgow. The groups studied were ABO, Rh CDE, Ms Ns, Pi, $Le^a$, K, $Fy^a$ and $JK^a$. There was no discordance between any of the twin members tested. The chances of dizygosity based on the twins being members of the same sex and the results of blood group analysis were low ranging from 2% to 14% This analysis did not take into account their physical appearance (other than sex), so clearly the possibilities of dizygosity were less than 2% to 14%.

3.8 Determination of E CR1 Number Using the Monoclonal Antibody E11

E CR1 number on the twins was measured by means of a RIA which employed the use of a radiolabelled monoclonal antibody to CR1, E11. Before E11 could be labelled the IgG fraction was isolated from the mouse ascites fluid; this fraction was then labelled with $^{125}I$ using Iodo-beads.

3.8(a) Isolation of IgG Fractions from Mouse Ascites Fluid (Steinbuch and Audran 1969)

Reagents

The monoclonal antibody anti-CR1 (E11) was a gift from Dr N Hogg, Cambridge, England.

Buffers

Acetic Acid (0.1 mol $l^{-1}$)

600µl of glacial acetic acid were added to 99.4mls of deionised water.

Saturated Ammonium Sulphate (SAS)

760g of ammonium sulphate were added to 1 litre of almost boiling deionised water. The solution was stirred until as much of the ammonium sulphate as possible had dissolved. The SAS was left overnight at room temperature and the pH was adjusted to 7.2 with sulphuric acid before use.

Procedure

The E11 mouse ascites fluid (1ml) was adjusted to pH 4.5 by addition of acetic acid (0.1 mol $l^{-1}$). Caprylic acid was then added such that a 5% (v/v) caprylic acid solution was obtained. The solution was shaken vigorously by hand and then centrifuged at 10,000g for 7 min at room temperature. The supernatant was carefully removed and the pellet discarded. While the supernatant was being stirred on a magnetic stirrer an equal volume of SAS was added dropwise to the supernatant. Stirring

was continued for 2 hours at room temperature to allow precipitation of IgG. The solution was then centrifuged at 10,000 for 7 min at room temperature and the supernatant removed. The pellet containing IgG was redissolved in 2mls of PBS. This was then placed into dialysis tubing and dialysed against 2 litres of PBS overnight at 4°C. Following this, protein concentration was determined spectrophotometrically by reading the optical density at 280nm and dividing this number by 1.43 which is the extinction coefficient for IgG. The protein solution was then divided into aliquots which were stored at -20°C.

The purified IgG fraction showed a single line of identity with rabbit anti-mouse IgG in double immunodiffusion agarose gels, a technique which has been described previously in chapter 2.6(f).

3.8(b) Radioiodination of E11 Using Iodo-Beads (Markwell 1982)

Each of the nonporous Iodo-beads are covalently modified with an oxidizing agent which facilitates the smooth and reproducible iodination of the tyrosine residues of peptides. This system of iodination is gentler than the chloramine-T method and it was therefore used to radiolabel the monoclonal anti-CR1 antibody E11.

Buffers

Sodium Phosphate Buffer

A stock solution of phosphate buffer was prepared as described in chapter 2.8. This was made to a pH of 7.4 and diluted to give a 0.05 mol $l^{-1}$ solution.

Procedure

The Iodo-beads were washed twice with sodium phosphate buffer, each time using 1ml of buffer per 2 beads. The beads were then dried on filter papers and added to 1mCi of carrier free $^{125}I$ which had been diluted with 200µl of phosphate buffer. The beads were incubated with the $^{125}I$ for 5 min at room temperature with occassional mixing by hand. E11 (1mg/ml) was then added to the preloaded Iodo-beads in the reaction vial (2 Iodo-beads were used to label 1mg of protein). A 15 min incubation at room temperature with occassional mixing by hand was the reaction time for maximal incorporation of $^{125}I$ into the protein. Following this incubation the bound and free iodine were separated by putting the reaction mixture over a 10ml Sephadex G-50 column which had been equilibrated with PBS. Gel filtration separates molecules on the basis of molecular weight thus the iodinated proteins, being heaviest, were eluted first from the column before the free iodine, thus separating the bound from the free iodine. Fractions (500µl) were collected and screened for $^{125}I$ content by counting a 1:100 dilution of each fraction in an automatic $\gamma$ counter (Fig 3.2). In this case fraction 6 and 7 were pooled and tested for precipitable counts by the acid precipitation technique

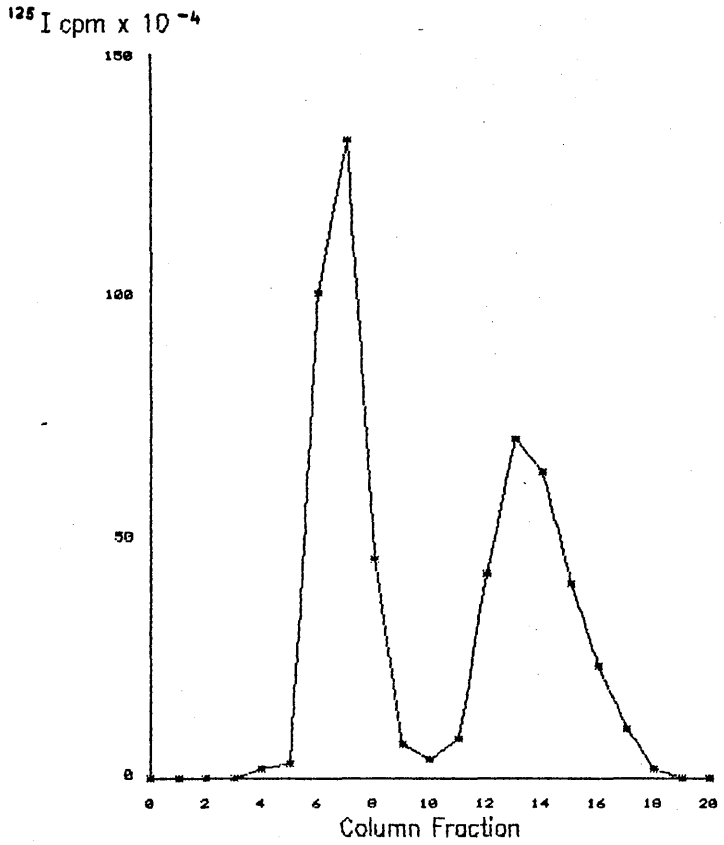

$^{125}$I cpm x $10^{-4}$
150
100
50
0
0
2
4
6
8
10
12
14
16
18
20
Column Fraction

Fig 3.2 Purification of $^{125}$I-Labelled E11 from Free Iodine

Legend

Purification of $^{125}$I-labelled IgG from free iodine using a 10ml Sephadex G-150 column. Fraction (500$\mu$l) were collected and the amount of $^{125}$I-cpm in a 1:100 dilution of each fraction was plotted against the fraction number. The first peak contained $^{125}$I-bound to E11 as determined by acid precipitation with TCA. Fractions 6 and 7 were pooled. The second peak contained free iodine.

which has been described in chapter 2.6(e). The percentage of acid precipitable counts was always greater than 95%. The protein concentration of the pool was then determined spectrophotometrically. This concentration was usually between 0.2 and 0.3mg/ml.

3.8(c) Quantification of CR1 sites/E using E11

Firstly the amount of CR1 specific IgG in the mouse ascites fluid was quantified. This was achieved by incubating a vast excess of E ($1x10^{10}$) with 250ng of $^{125}$I-E11-IgG in the presence and absence of a 100-fold molar excess of unlabelled E11-IgG. This experiment was performed with cells from 3 individual donors. The mean percentage of specific binding of E11-IgG was 32%. It was therefore decided to add 700ng of purified E11-IgG to $5x10^{7}$E. Of this 32% was capable of binding to CR1 which represents 224ng of specific E11-IgG which was sufficient to saturate all available CR1 sites.

Reagents

Dibutylphthalate/Dinonylphthalate

60mls of dibutylphthalate were mixed with 40mls of dinonylphthalate.

Procedure

Triplicate aliquots (200µl) of E ($2.5x10^{8}$/ml) in RPMI/BSA were transferred to microcap tubes. To each tube 700ng of $^{125}$I-E11 were added and then the E were incubated for 1 hour at 4°C with end over end mixing on a Matburn mixer. In order to assess nonspecific binding of $^{125}$I-E11, a 10-fold molar excess of unlabelled E11 was added to separate triplicate aliquots (200µl) of E ($2.5x10^{8}$/ml). These cells were incubated for 30 min at 4°C with end over end mixing on a Matburn mixer before addition of 700ng of $^{125}$I-E11 and incubation for a further 1 hour at 4°C. When the incubation periods were complete the E were layered onto 300µl of dibutylphthalate/dinonylphthalate in microcap tubes and the bound and free $^{125}$I-E11 separated by centrifugation at 10,000g for 2 min at room temperature. The supernatant was removed and the red cell pellet was cut from the bottom of the tube using a hot scalpel blade. The pellets containing $^{125}$I-E11 bound to E were placed into glass carrier tubes and counted in an automatic $\gamma$ counter.

3.8(d) Calculation of E CR1 Number

The amount of radioactivity bound in the absence of unlabelled E11 represents the total amount of $^{125}$I-E11 bound to the E. The amount of radioactivity bound in the presence of unlabelled E11 represents the nonspecific binding of $^{125}$I-E11. The specific number of bound cpm were obtained by subtracting the mean of the nonspecific binding from the mean of the total binding. The number obtained represented the number of

bound cpm to $5x10^7$E. To convert the bound cpm into CR1 antigenic sites per cell the following calculations were performed.

The number of molecules in 1cpm =

$$\frac{2.8 \times 10^{12}}{\text{Number of cpm in 700ng of IgG anti-CR1}}$$

The number of antigenic sites per cell =

$$\frac{\text{The number of molecules in 1cpm} \times \text{The number of bound cpm in } 5x10^7\text{E}}{5x10^7}$$

The explanation for this calculation is as follows, 1 mole of IgG contains 150,000g or $6.03 \times 10^{23}$ molecules (Avagadro's number), therefore 700ng of IgG molecules contains $2.82x10^{12}$ molecules. For each assay the number of molecules in 1cpm were determined by dividing $2.82x10^{12}$ by the number of cpm in 700ng of IgG anti-CR1. This input at 700ng varied for each assay according to the natural decay of the $^{125}$I label. Having obtained the number of molecules in 1cpm it was then possible to calculate the number of molecules contained in the number of bound cpm which was for the total E input of $5x10^7$/tube. Division of this number by $5x10^7$ resulted in the number of CR1 molecules (antigenic sites) per E.

3.9 Control for E Lysis During Determination of E CR1 Number

As already mentioned (3.6) the thawing process resulted in a variable degree of lysis. Although before standardisation each sample was given 5 washes in RPMI until no further lysis occured, during the assay procedure itself additional lysis occurred. To control for this a 1ml E sample ($2.5x10^8$/ml) from each twin was placed into a microcap tube and subjected to the same conditions as the test samples. After centrifugation over dibutylphthalate/dinonylphthalate a 100µl aliquot of the supernatant was removed and added to 2.9mls of deionised water and the $OD_{541}$ was measured. The number of lysed E were then calculated and subtracted from the total input into each microcap tube. This number was then used in the calculation described in 3.8(d) instead of $5x10^7$.

Electron microscopy of the E pellet revealed that the membranes from the lysed E were not pelleted out during the centrifugation procedure and therefore did not contribute to the final E CR1 number.

3.10 Validity of CR1 Number after Freeze/Thawing

To ensure that the freezing and thawing process did not result in alterations of E CR1 number the following control experiments were performed. Venous blood (20mls) was collected from 5 donors; this was divided into 2 10ml aliquots. The E from 1 of the aliquots from each donor was washed and standardised to $2.5x10^8$/ml and E CR1 number

determined. The remaining aliquot from each donor was frozen and stored at -70°C. Following storage the E were thawed and CR1 number determined.

3.11 Intra-Assay Variation

In order to assess the reproducibility of the assay a blood sample was taken from 1 individual and divided into 3 aliquots. Each aliquot was treated as a separate sample and the E CR1 number for each was calculated. All of the assays were performed at the same time. Table 3.1 shows that for 3 donors the greatest variation was ±9% from the mean.

3.12 Inter-Assay Variation

In order to assess the inter-assay variation, aliquots of frozen red cell pellets from the same twin sample were thawed out on 2 separate occassions and E CR1 number determined. As is shown in Table 3.2 the greatest inter-assay variation for 6 donors was ±7% from the mean.

3.13 Enzyme Linked Immunosorbent Assay (ELISA)

The basis for this method involves the attachment of antibody to plastic microtitre plates. Following blockade of any free binding sites left on the microtitre plate with BSA, antigen was added to the wells. Antigen bound to antibody is detected by the addition of a second antibody to the antigen labelled with horseradish peroxidase (HRP). This enzyme catalyses the reduction of hydrogen peroxide and oxidation of OPD from a clear to a coloured solution. The intensity of colour, which is an indication of antigen concentration, is measured spectrophotometrically at an absorbance of 492nm on an ELISA reader.

Reagents

Coating Antibodies

Coating antibodies were donated by Dr L Morrison. They were stored at -20°C and contained 0.01% thiomersal as a preservative. Prior to use they were diluted in coating buffer to the concentrations indicated below.

Anti-H (5μg/ml)

Anti-C4bp (10μg/ml)

Anti-I (10μg/ml)

Conjugates

All conjugates were kindly donated by Dr L Morrison, they were stored at 4°C and contained 0.01% thiomersal as a preservative. Prior to use they were diluted in 0.05%(v/v) Tween/PBS to the concentrations indicated below:

Table 3.1 Intra-assay Variation

CR1 Sites/E

| Sample | Aliquot 1 | Aliquot 2 | Aliquot 3 | Mean±SD | % Variation |
|---|---|---|---|---|---|
| 1 | 271 | 313 | 295 | 293±21 | +7 -7 |
| 2 | 1105 | 1096 | 953 | 1051±85 | -9 +5 |
| 3 | 1046 | 1100 | 1023 | 1056±39 | -3 +4 |

Note: The mean of each set of 3 measurements was calculated. The difference between the 2 extremes and the mean in each set was then expressed as a percentage of the mean.

Table 3.2 Inter-assay Variation

CR1 Sites/E

| Sample | 1st Measurement | 2nd Measurement | Mean | % Variation from the mean |
|---|---|---|---|---|
| 1 | 618 | 683 | 650 | +5 -5 |
| 2 | 1856 | 2117 | 1986 | +7 -7 |
| 3 | 752 | 754 | 753 | 0 |
| 4 | 1536 | 1385 | 1460 | +5 -5 |
| 5 | 635 | 705 | 670 | +5 -5 |
| 6 | 953 | 927 | 940 | +1 -1 |

Note: The mean for each 2 measurements was calculated. Each number was then expressed as a percentage of this mean.

HRP anti-C4bp (diluted 1:250)

HRP anti-H (diluted 1:10,000)

HRP anti-I (diluted 1:100)

Standard

A serum standard consisting of a pool of serum from 50 normal donors was used in each assay. This standard contained H (300µg/ml), C4bp (250µg/ml) and I (50µg/ml). This standard was diluted in Tween/PBS from 1:500 to 1:512,000 for the H ELISA, from 1:2,000 to 1:128,000 for the C4bp ELISA and from 1:20 to 1:5120 for the I ELISA.

Twin Serum Samples

The twin serum samples were diluted in 0.05%(v/v) Tween/PBS to the dilutions indicated below.

C4bp (1:8,000), H (1:32,000) and I (1:320)

Substrate

17mg of OPD were dissolved in 50mls of phosphate/citrate buffer. Immediately before use 20µl of hydrogen peroxide ($H_2O_2$) were added to this solution.

Buffers

Tween/PBS 0.05%(v/v)

0.5mls of Tween 20 were added to 1 litre of PBS.

Coating Buffer(carbonate/bicarbonate buffer (0.05 mol $l^{-1}$ pH 9.6))

0.79g of sodium carbonate and 1.46g of sodium hydrogen carbonate were made up to a final volume of 500mls with deionised water.

Blocking Buffer

0.5g of BSA were added to 500mls of PBS.

Phosphate/Citrate Buffer

11mls of citric acid (0.1 mol $l^{-1}$) were mixed with 14mls of disodium hydrogen phosphate (0.2 mol $l^{-1}$) to give a final pH of 5.6, 25mls of deionised water were then added.

Procedure

A microtitre plate containing 96 wells was coated with 100µl of the IgG fraction of the antisera at the concentrations indicated. The plate was incubated overnight at 4°C in a humid atmosphere. The following morning the plate was aspirated and washed 5 times with Tween/PBS ensuring that all the wells were filled to the top with each wash. Any remaining free sites on the microtitre plate were blocked by incubation with 250µl of PBS/BSA for 1 hour at room temperature in a humid atmosphere. The plate was aspirated and washed 5 times with Tween/PBS and then duplicate aliquots (100µl) of the serum standard at the dilutions indicated were added to the top 2 rows of wells. Triplicate aliquots (100µl) of a suitable dilution of the twin sera were then added to the remaining wells and the plate was incubated at room temperature for 2 hours in a humid atmosphere. The plate was then aspirated and washed 5 times with Tween/PBS and 100µl of the HRP-conjugated IgG fraction of the same antiserum used for coating the wells was added and the plate was incubated at room temperature for 1 hour in a humid atmosphere. The plate was aspirated and washed 10 times with Tween/PBS and 100µl of the substrate was added. The plate was incubated for 30 min in the dark in a humid atmosphere, before addition of 25µl of sulphuric acid (4 mol $l^{-1}$) to each well to stop the reaction. The absorbance of each well at 492nm was then read using an automatic ELISA reader.

## 3.14 Calculation of Serum Concentrations of C4bp, H and I

The concentration of each particular protein in the standard serum pool was calculated for each dilution of the standard curve. This was plotted onto semilog paper against the mean of the $OD_{492}$ obtained from the ELISA assay for the standard dilutions (Fig 3.3). The $\log_{10}$ values of the serum concentrations were obtained and these values were used as the x-coordinates and the $OD_{492}$ of each of the dilution were used as the y-coordinates in a linear regression analysis. If the correlation coefficient of the line obtained was greater than 0.995 then this line was used to determine the serum concentrations of the particular protein under study. If the correlation coefficient was lower than 0.995 then any points on the graph which deviated from the straight line were omitted from the calculation. Usually the highest and lowest dilutions were omitted as the line tended to curve at its upper and lower limits. If the $OD_{492}$ of the sample fell outwith the linear portion of the standard curve the assay was repeated using a more appropriate serum dilution.

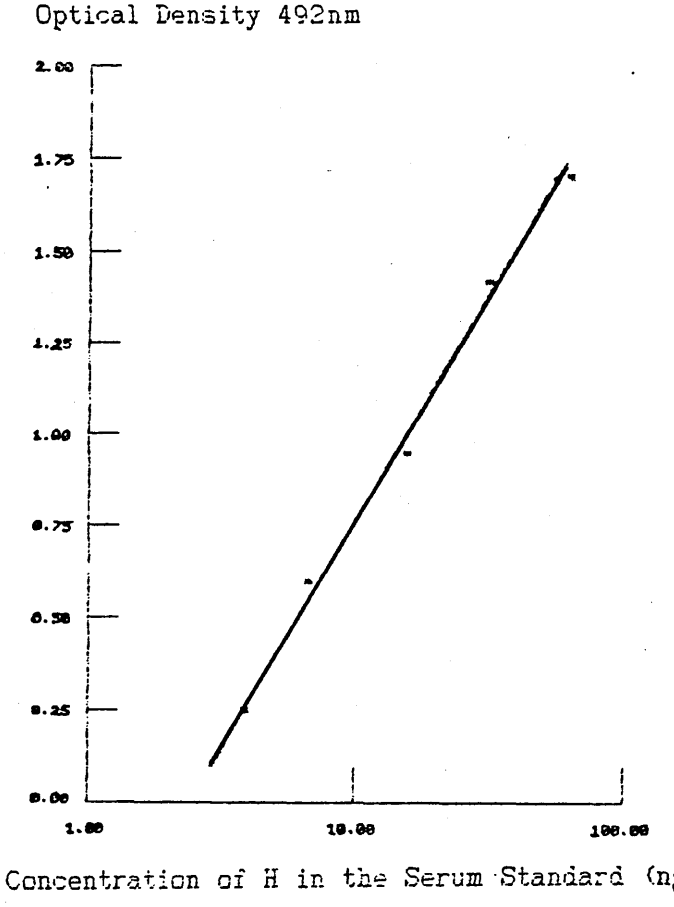
## 3.15 Intra-Assay Variation for the ELISA Assay

The intra-assay variation for the different ELISA procedures was ±5% of the mean.

Fig 3.3 Standard Curve of Serum Pool

Legend

A standard curve was constructed by plotting the $OD_{492}$ of a dilution of the standard containing a known concentration of H against that concentration. The linear regression analysis performed on these points gave a correlation coefficient of 0.996.


Optical Density 492nm
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1.00
10.00
100.00
Concentration of H in the Serum Standard (ng)

## 3.16 Inter-Assay Variation for the ELISA Assay

The inter-assay variation for all the proteins tested was ±6% of the mean.

## 3.17 Statistical Analysis

### 3.17(a) Genetic Analysis

The most commonly employed techniques in the genetic analysis of continuous characteristics are the study of intra(within)-pair and inter(between)-pair variances (Osborne and De George 1959) and the intra-class correlation coefficient.

#### The Intra-pair Variance

The intra-pair variance is calculated by the equation shown below (which has N degrees of freedom):

$$\frac{\Sigma\ (A-B)^2}{2N}$$

where A and B are the assay results for a pair of twins and $\Sigma$ donates summation over a set of N pairs.

#### The Inter-pair Variance

The inter-pair variance (which usually has N-1 degrees of freedom) is calculated by the equation shown below:

$$\frac{1}{DF}\left[\frac{\Sigma\ (A+B)^2}{2} - \frac{\Sigma\ (A+B)^2}{2N}\right]$$

where DF, the degrees of freedom is at most equal to N-1. However when corrections were made for batch effect several degrees of freedom were lost and this affected the calculation.

Variances were compared by dividing the larger by the smaller, the ratio being referred to as 'F', the statistical significance of which can be determined from standard tables of F values.

#### Intra-class Correlation Coefficient (r)

In measuring correlations between twins it is not possible to decide which measurement on a pair of twins is x and which is y. For this reason, the intra-class correlation coefficient (r), which treats each

pair of twins symmetrically, is preferred to the most usual inter-class correlation coefficient. It is equal to:

$$\frac{\text{Inter-pair Variance} - \text{Intra-pair Variance}}{\text{Inter-pair Variance} + \text{Intra-pair Variance}}$$

3.17(b) Batch Effect

When E CR1 numbers and serum concentrations of H and C4bp were measured, each member of a twin pair was measured in the same batch. This may have led to similarities between twins which were purely as a result of the twins being in the same batch. Statistical tests were therefore performed to determine if there was a batch effect. One way analysis of variance showed that there was a significant difference between batches for each of the 3 proteins. Therefore each measurement was first corrected by subtracting its corresponding batch mean and adding to this number the grand mean of all the measurements for that particular protein. These corrected numbers were subsequently used to test for age effects and for correlations between sets and subsets of twins.

3.17(c) Age Effect

A pair of twins may resemble each other not only because they have the same genes but also because they have the same age. The correlations of H, C4bp and I levels with age were significant, with serum concentrations of each increasing with age. It was therefore necessary to age-adjust the measurements to ensure that age effects would not contribute to any correlation between the twins. This was achieved by calculating the increase observed each year and subtracting this from the measurements for each year above the mean age of the population studied and adding the increase on for every year below the mean age.

3.17(d) Mean Values

In this analysis the geometric mean values are quoted as opposed to the arithmetic mean values. The geometric mean value is the anti-log of the mean value of the $\log_{10}$ value of each protein. This is more representative of a positively skewed distribution than is the arithmetic mean, because the latter is very sensitive to measurements at the high end of a skewed distribution.

## Results

### 3.18 Validity of CR1 Number after Freeze/Thawing

Before any of the twin blood samples were collected and frozen it was first of all established that the freezing and thawing process did not alter E CR1 expression. Table 3.3 shows the number of CR1 sites/E calculated on unfrozen blood samples and on the same sample after it had been frozen and thawed. As can be seen from the table the largest percentage variation in CR1 number was ±6% of the mean.

### 3.19 Distribution of CR1 Sites/E in all of the Twins Studied

Using the raw data a histogram was constructed showing the distribution of CR1 sites/E in all of the twins studied (1 pair of twins is missing in all of the CR1 data) (Fig 3.4(a)). As can be seen from Fig 3.4(a) this approximates to a log-normal distribution. For the purposes of statistical analysis it was necessary to transform each of the CR1 measurements to the $\log_{10}$ value (Fig 3.4(b)). The anti-log geometric mean of CR1 sites/E was 837.

### 3.20 Comparison of CR1 Measurements in Males and Females

After correction for batch effect the geometric mean E CR1 values for males and females were compared to determine if there was a relationship between CR1 and sex. The geometric mean for males was 839 and that for females was 829. As these mean values were almost identical it may be concluded that sex does not influence the mean value of E CR1.

### 3.21 Effect of Age on CR1

The CR1 numbers from all the twins were used to test for age effects. The correlation coefficient between CR1 and age was not significant. Therefore it may be concluded that there is no relationship between CR1 and age.

### 3.22 Genetic Analysis of CR1 Sites/Erythrocyte

The intra-pair and inter-pair variances and the intra-class correlation coefficients were calculated for all the sets and subsets of twins. The results are shown in Table 3.4.

None of the F values quoted in Table 3.4 are significant (apart from the F value for the batch variation). This indicates that the differences between dizygotic twins were no greater than the differences between monozygotic twins. There is also no evidence of any correlations between any of the sets or subsets of twins (Fig 3.5(a),(b) and (c)). Thus it may be concluded that the genetic influence on E CR1 numbers in the normal population, if it exists, was too small to detect. Differences between twins must therefore arise largely as a result of environmental factors.

Table 3.3 Comparison of CR1 Sites/E Before and After Freeze/Thawing

| Donor | Unfrozen Blood | Frozen Blood | Mean | % Variation from the Mean |
|---|---|---|---|---|
| 1 | 466 | 473 | 469 | +1 -1 |
| 2 | 333 | 337 | 335 | +1 -1 |
| 3 | 258 | 229 | 243 | +6 -6 |
| 4 | 338 | 362 | 350 | +3 -3 |
| 5 | 219 | 214 | 216 | +1 -1 |

Note: CR1 number was calculated on freshly isolated E. An aliquot of E from the same donor was then frozen and stored at -70°C. The blood was thawed and CR1 number determined taking into account the degree of lysis. The mean of both determinations was calculated for each set of measurements. Both measurements were then expressed as a percentage of the mean.

Fig 3.4(a) Distribution of CR1 Sites/E

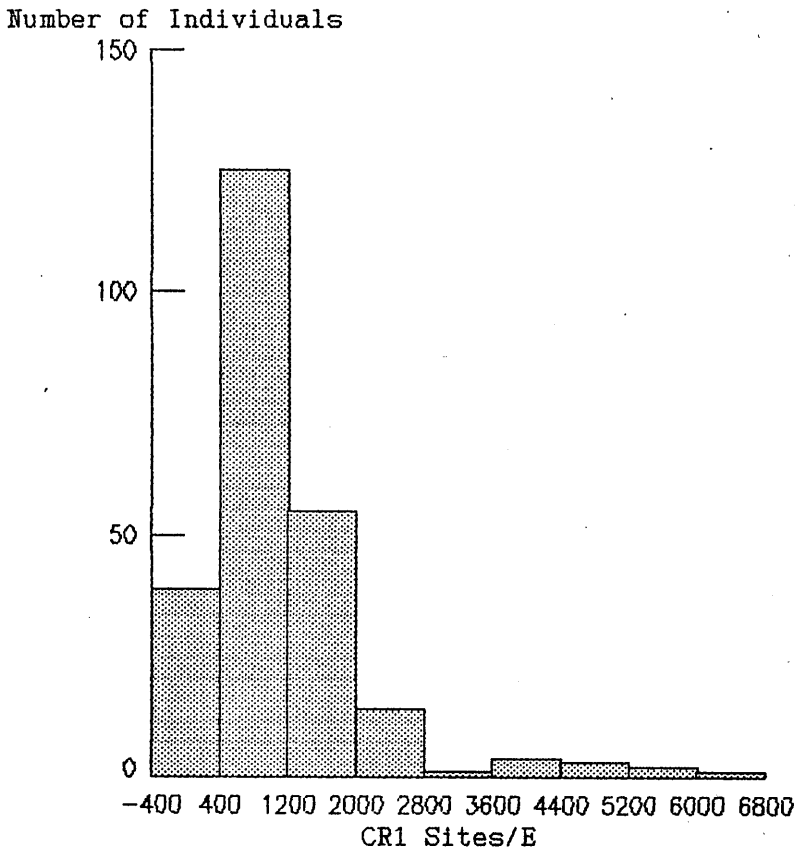
Legend

Frequency histogram showing the distribution of CR1 sites/E on 122 pairs of twins.

Fig 3.4(b) Distribution of $\log_{10}$ Transformed CR1 Sites/E

Legend

Frequency histogram showing the distribution of CR1 sites/E in 122 pairs of twins after transformation of each to the $\log_{10}$.


Number of Individuals
150
100
50
0
-400 400 1200 2000 2800 3600 4400 5200 6000 6800
CR1 Sites/E

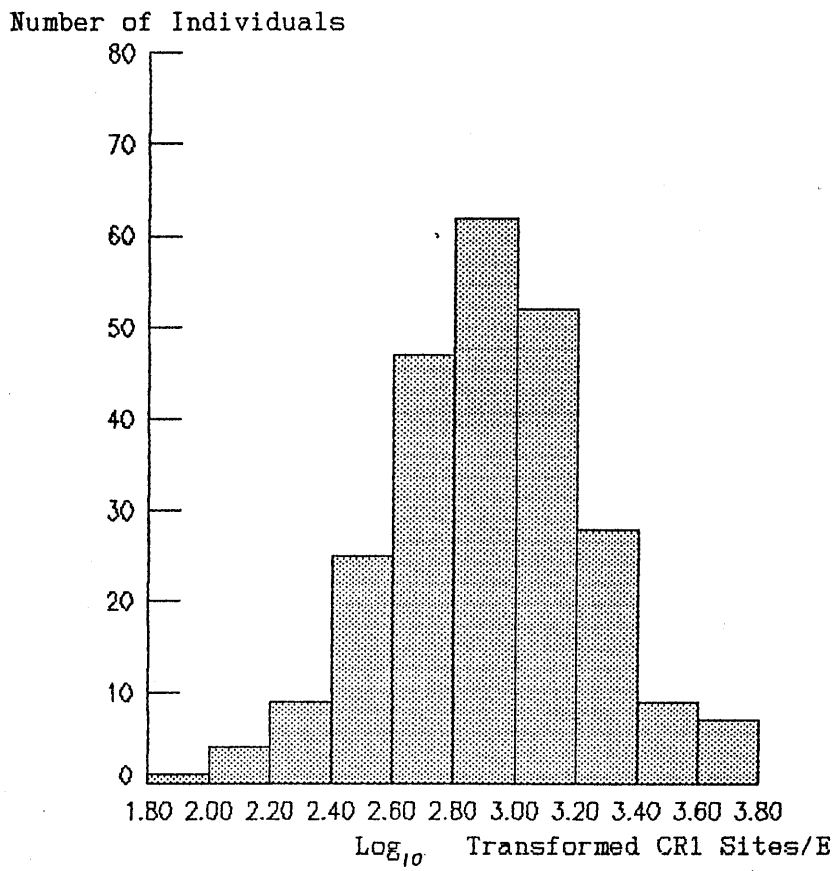

Number of Individuals
80
70
60
50
40
30
20
10
0
1.80 2.00 2.20 2.40 2.60 2.80 3.00 3.20 3.40 3.60 3.80
Log10 Transformed CR1 Sites/E

Table 3.4 Analysis of Variance of $Log_{10}$ CR1 Sites/E

Notes

+ This figure was obtained by subtracting each individual measurement from the grand mean of all the measurements, squaring this figure and then adding up all the squares. It gives an indication of what the variation from the mean is in each of the groups indicated under source of variance.

· The degrees of freedom for the inter-pair variation are low because of correction for batch effect. This correction is not necessary for intra-pair variation as the pairs of twins were measured in the same batch and therefore the difference between one individual and his twin is always the same.

[] The mean square is obtained by dividing the sum of squares by the degrees of freedom. This figure corresponds to the intra-pair or inter-pair variance.

* Variances were compared by dividing the inter-pair mean square by each mean square the ratio being referred to as F. This figure indicates whether the difference within a pair of twins is significantly different from the differences between pairs of twins.

# This F value is significant. This indicates that there was a significant difference between batches, larger than might be expected by random sampling error. Correction for batch effect was necessary because of this.

NS = Not Significant

DF = Degrees of Freedom

F = Female

M = Male

Table 3.4 Analysis of Variance of $Log_{10}$ CR1 Sites/E

| Source of Variance | Sum of+ Squares | DF° | Mean [] Square | F Ratio* | Significance |
|---|---|---|---|---|---|
| Total | 26.4236 | 243 | | | |
| Batch Effects | 11.4381 | 40 | 0.2859 | 3.644* | P < 0.001 |
| Age Effects | 0.0012 | 1 | 0.00116 | 67.269 | NS |
| Inter-pair | 6.24256 | 80 | 0.78032 | | |
| Intra-pair | 8.74181 | 122 | 0.071654 | 1.089 | NS |
| ...divided as follows | | | | | |
| Monozygotic | 5.00698 | 60 | 0.083450 | 1.069 | NS |
| Dizygotic | 3.73483 | 62 | 0.060239 | 1.295 | NS |
| ...and subdivided as follows | | | | | |
| Monozygotic F | 4.54759 | 53 | 0.085804 | 1.100 | NS |
| Monozygotic M | 0.459390 | 7 | 0.065627 | 1.189 | NS |
| Dizygotic F-F | 2.78563 | 35 | 0.079589 | 1.020 | NS |
| Dizygotic M-F | 0.801119 | 21 | 0.038149 | 2.045 | NS |
| Dizygotic M-M | 0.148083 | 6 | 0.024681 | 3.162 | NS |

Fig 3.5(a) Correlations Between CR1 Levels in Twins

Legend

Scatter graph showing a plot of $\log_{10}$ E CR1 levels in twin 2 against twin 1. The correlation coefficient was not significant.

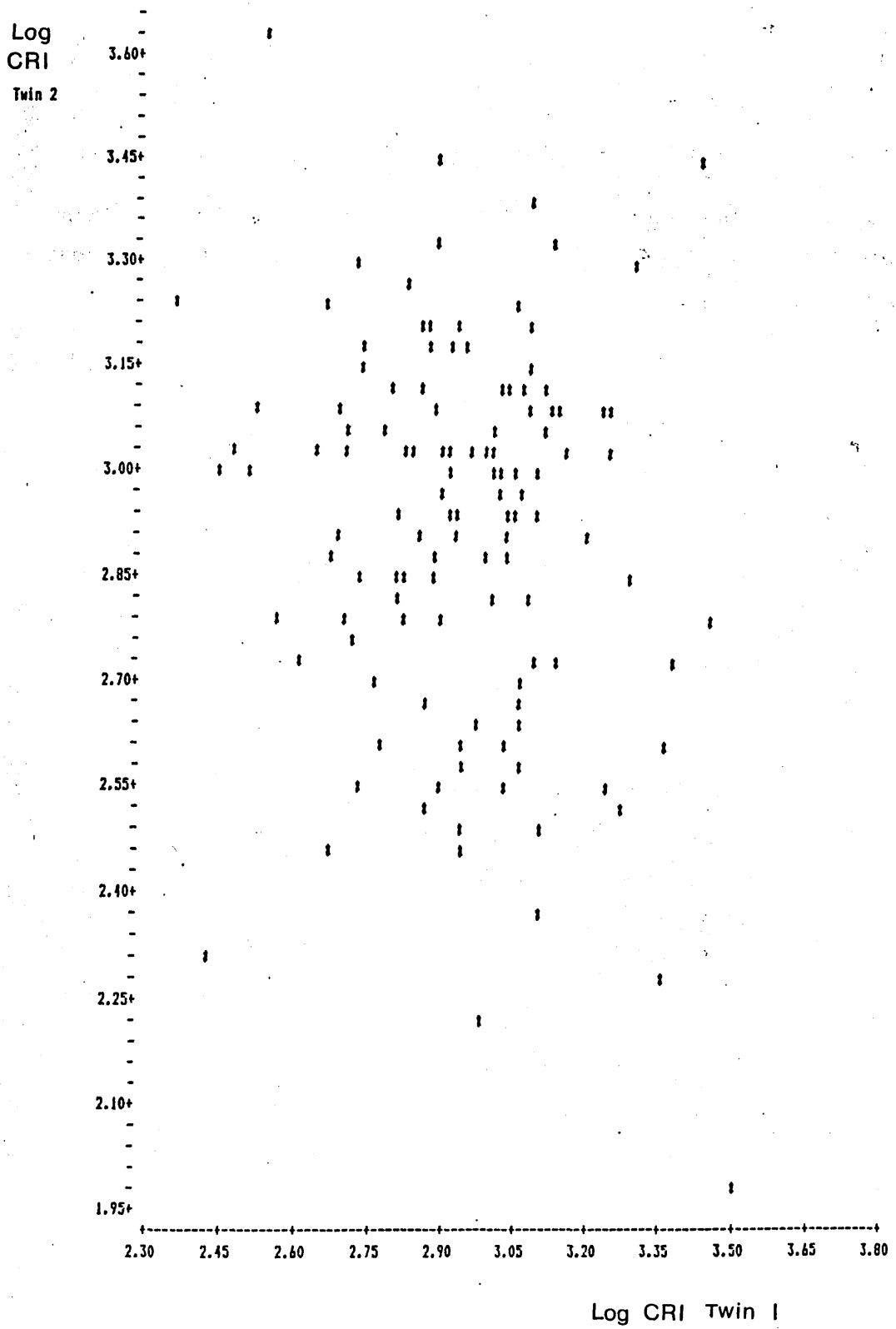

Log
CRI
Twin 2
3.60+
3.45+
3.30+
3.15+
3.00+
2.85+
2.70+
2.55+
2.40+
2.25+
2.10+
1.95+
2.30
2.45
2.60
2.75
2.90
3.05
3.20
3.35
3.50
3.65
3.80
Log CRI Twin 1

Fig 3.5(b) Correlations Between CR1 Levels in Twins

Legend

Scatter graph showing a plot of $\log_{10}$ E CR1 levels in identical twins only. Twin 2 is plotted against twin 1. Again the correlation coefficient was not significant.

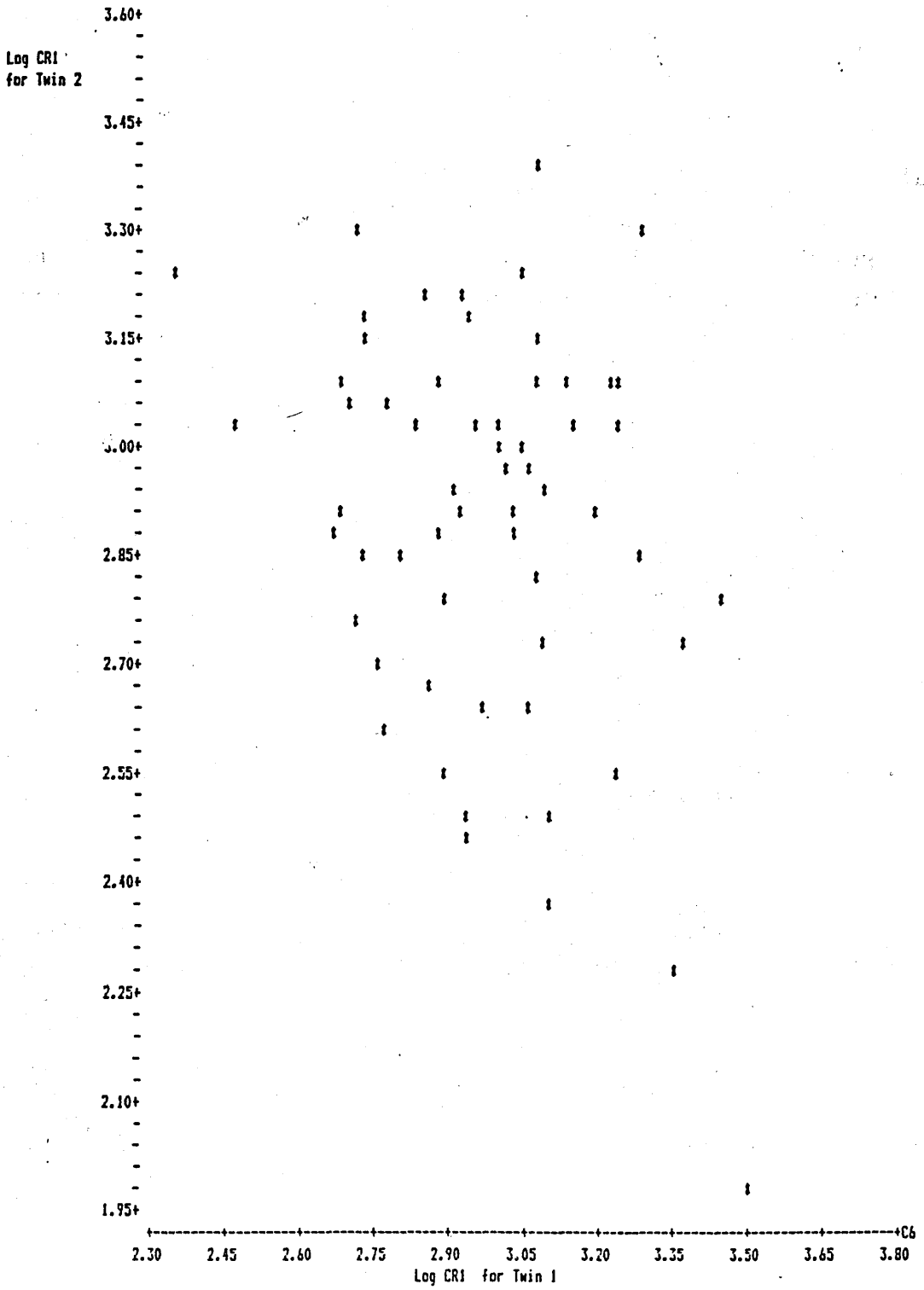
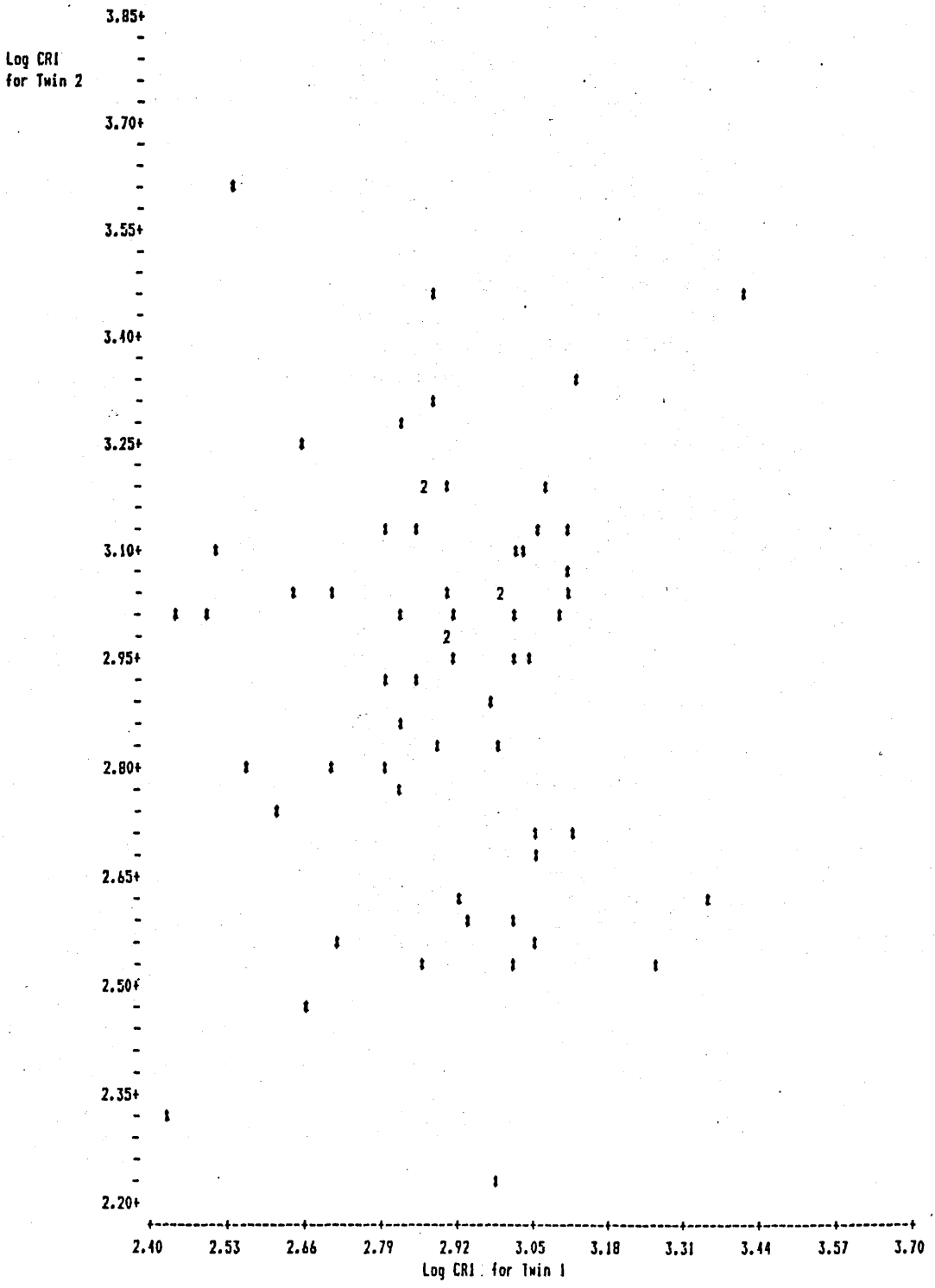

Log CRI for Twin 2
3.60+
3.45+
3.30+
3.15+
3.00+
2.85+
2.70+
2.55+
2.40+
2.25+
2.10+
1.95+
2.30
2.45
2.60
2.75
2.90
3.05
3.20
3.35
3.50
3.65
3.80
Log CRI for Twin 1

Fig 3.5(c) Correlations Between CR1 Levels in Twins

Legend

Scatter graph showing a plot of $\log_{10}$ E CR1 levels in nonidentical twins only. Twin 2 is plotted against twin 1. The correlation coefficient was not significant.


Log CRI
for Twin 2
3.85+
3.70+
3.55+
3.40+
3.25+
3.10+
2.95+
2.80+
2.65+
2.50+
2.35+
2.20+
2.40
2.53
2.66
2.79
2.92
3.05
3.18
3.31
3.44
3.57
3.70
Log CRI for Twin 1

3.23 Analysis of Serum Concentrations of H in Twins

The distribution of H levels in all of the twins studied is shown in Fig 3.6(a). The distribution shows a strong positive skew. As in the CR1 assay the H levels had to be transformed to a normal distribution for the purposes of statistical analysis. This was achieved by subtracting 80 from each of the original measurements and taking the $\log_{10}$ of this number ($\log_{10}$(H-80)) (Fig 3.6(b)). The mean value of $\log_{10}$(H-80) in all the samples tested was 2.39, which corresponds to a concentration of 324μg/ml.

3.24 Effect of Age on Serum Concentration of H

The correlation between transformed H levels and age was 0.43. This number is significant ($p < 0.005$) (Fig 3.7). Thus H levels do appear to increase significantly with age. The data was therefore corrected for both batch and age effect as described earlier and analysed for differences between males and females.

3.25 Comparison of H Levels in Males and Females

To determine if males and females have different amounts of H, the mean values of H were calculated for males and females. The mean for males was 341μg/ml and the mean for females was 320μg/ml. As there was no significant difference between the two it may be concluded that there is no difference in serum concentrations of H between males and females.

3.26 Genetic Analysis of H Levels

The data were corrected for both batch effect and age affect and the results of the analysis of variance are shown in Table 3.5.

There was some resemblance between twins of various sets and subsets, as seen by the fact that the intra-pair variances were generally smaller than the inter-pair variance. These resemblances showed moderate degrees of statistical significance as is indicated in Table 3.5. However, when the F ratio to compare dizygotic intra-pair variance (0.03005) with monozygotic intra-pair variance (0.02540) was calculated the value obtained 1.18311, was not at all significant ($P > 0.1$). There was therefore no evidence at all of any genetic influence on H levels.

3.27 Analysis of C4bp Measurements in Twins

Once again there was a strong positive skew in C4bp distribution when the raw data were plotted as a frequency histogram (Fig 3.8(a)). The distribution was made almost normal by subtracting 85 from each of the original measurements and taking the $\log_{10}$ of this number ($\log_{10}$(C4bp-85)).(Fig 3.8(b)). The mean of this transformed data was 1.943, which corresponds to a C4bp concentration of 173μg/ml.

Fig 3.6(a) Distribution of H levels

Legend

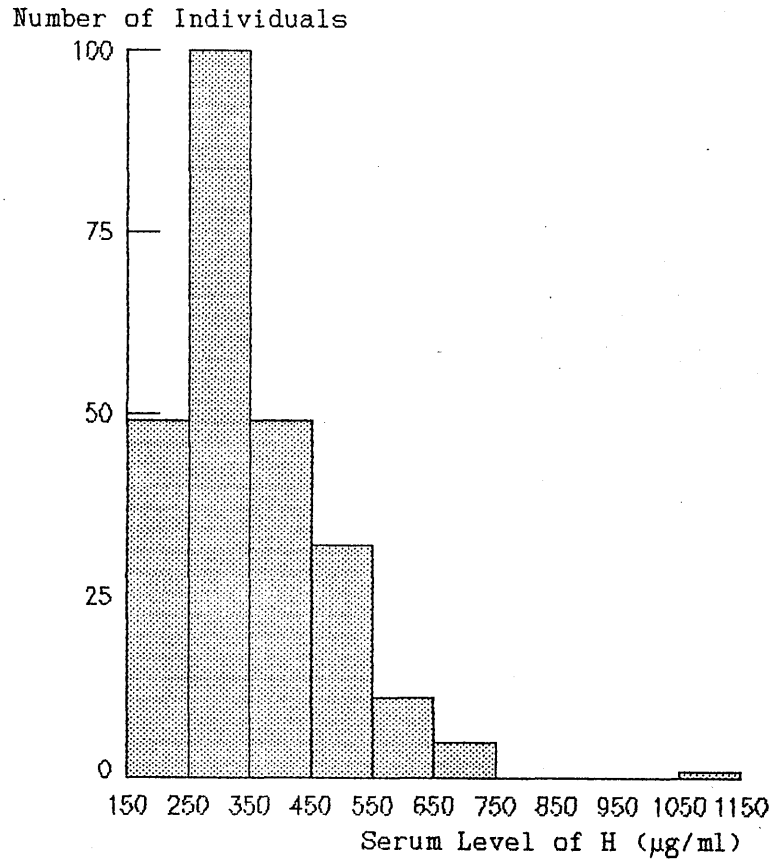
Frequency histogram showing the distribution of serum levels of H in 123 pairs of twins.

Fig 3.6(b) Distribution of $Log_{10}$ (H-80) Levels

Legend

Frequency histogram showing the distribution of serum levels of H in 123 pairs of twins after transformation of each to $log_{10}$ (H-80).

(a)


Number of Individuals
100
75
50
25
0
150 250 350 450 550 650 750 850 950 1050 1150
Serum Level of H (μg/ml)

(b)

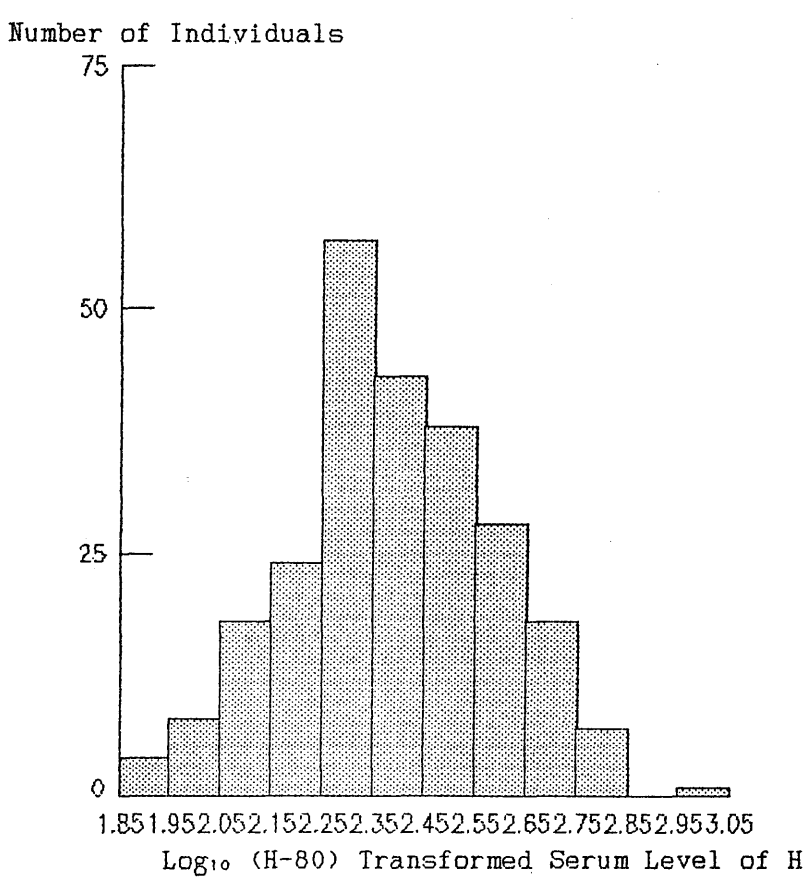

Number of Individuals
75
50
25
0
1.85 1.95 2.05 2.15 2.25 2.35 2.45 2.55 2.65 2.75 2.85 2.95 3.05
Log10 (H-80) Transformed Serum Level of H

Fig 3.7 Correlation Between H Levels and Age

Legend

Scatter graph showing a plot of serum concentration of H against the age of the twin. The correlation coefficient was 0.43 which is significant.

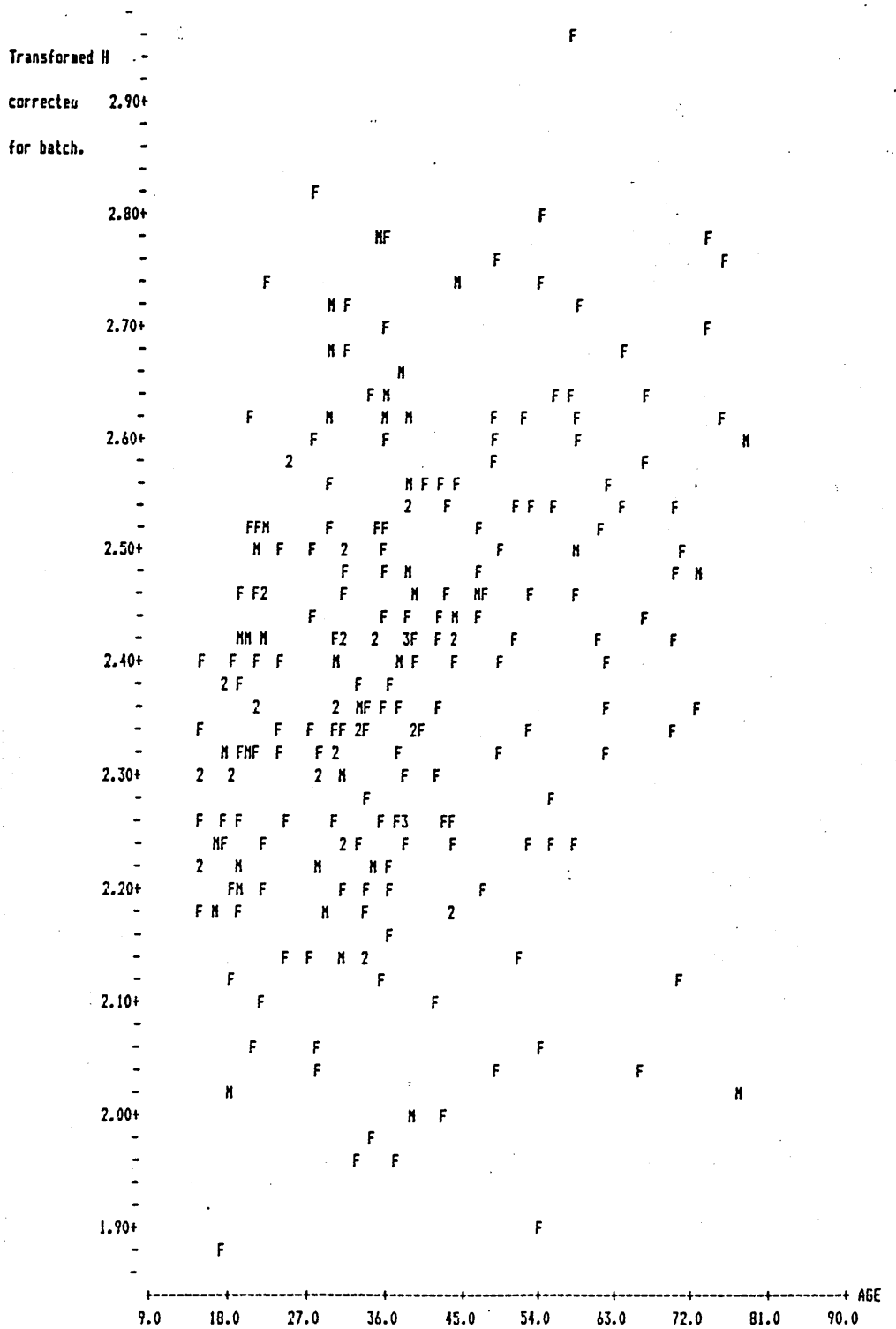

Transformed H
corrected
for batch.
2.90+
2.80+
2.70+
2.60+
2.50+
2.40+
2.30+
2.20+
2.10+
2.00+
1.90+
+---------+---------+---------+---------+---------+---------+---------+---------+---------+ AGE
9.0 18.0 27.0 36.0 45.0 54.0 63.0 72.0 81.0 90.0

Table 3.5 Analysis of Variance of $Log_{10}$ (H-80) Levels

| Source of Variance | Sum of Squares | DF | Mean Square | F Ratio | Significance |
|---|---|---|---|---|---|
| Total | 9.65146 | 245 | | | |
| Batch Effect | 0.686055 | 8 | 0.0857569 | 1.9696 | P ~ 0.05 |
| Age Effect | 0.62553 | 1 | 0.62553 | 14.3668 | P < 0.005 |
| Inter-pair | 4.92002 | 113 | 0.04354 | | |
| Intra-pair | 3.41256 | 123 | 0.02774 | 1.5696 | P < 0.01 |
| ...divided as follows | | | | | |
| Monozygotic | 1.54919 | 61 | 0.0254 | 1.7142 | P <0.025 |
| Dizygotic | 1.86337 | 62 | 0.03005 | 1.4489 | NS |
| ...subdivided as follows | | | | | |
| Dizygotic F | 1.1310 | 35 | 0.03231 | 1.3476 | NS |
| Dizygotic M/F | 0.47144 | 21 | 0.02245 | 1.9394 | P<0.05 |
| Dizygotic M | 0.26093 | 6 | 0.04349 | 1.0001 | NS |

Fig 3.8(a) Distribution of C4bp Levels

Legend

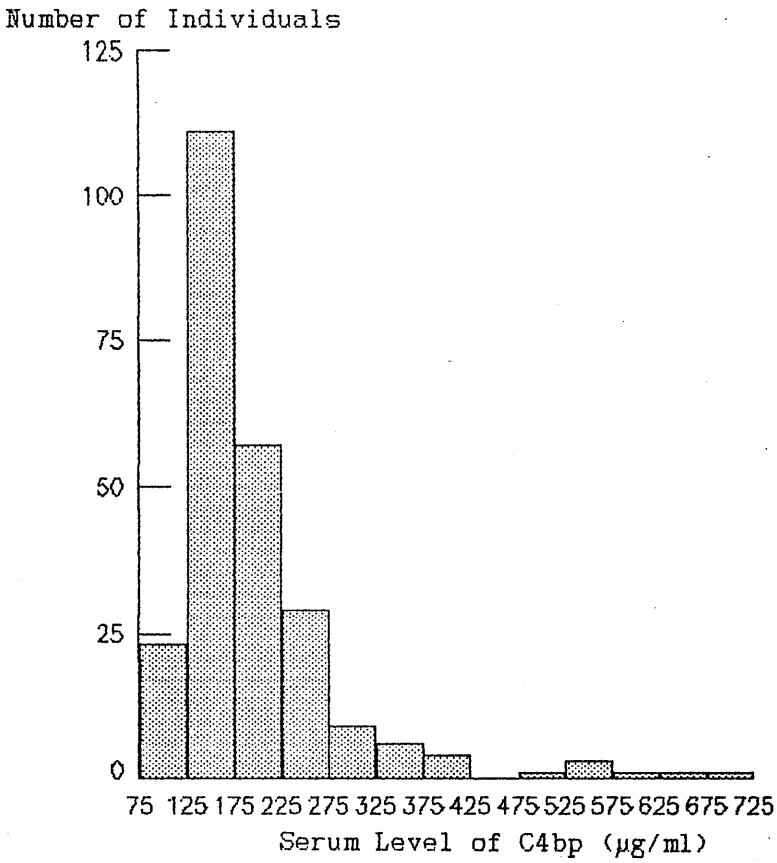
Frequency histogram showing the distribution of serum levels of C4bp in 123 pairs of twins.

Fig 3.8(b) Distribution of $Log_{10}$ (C4bp-85) Levels

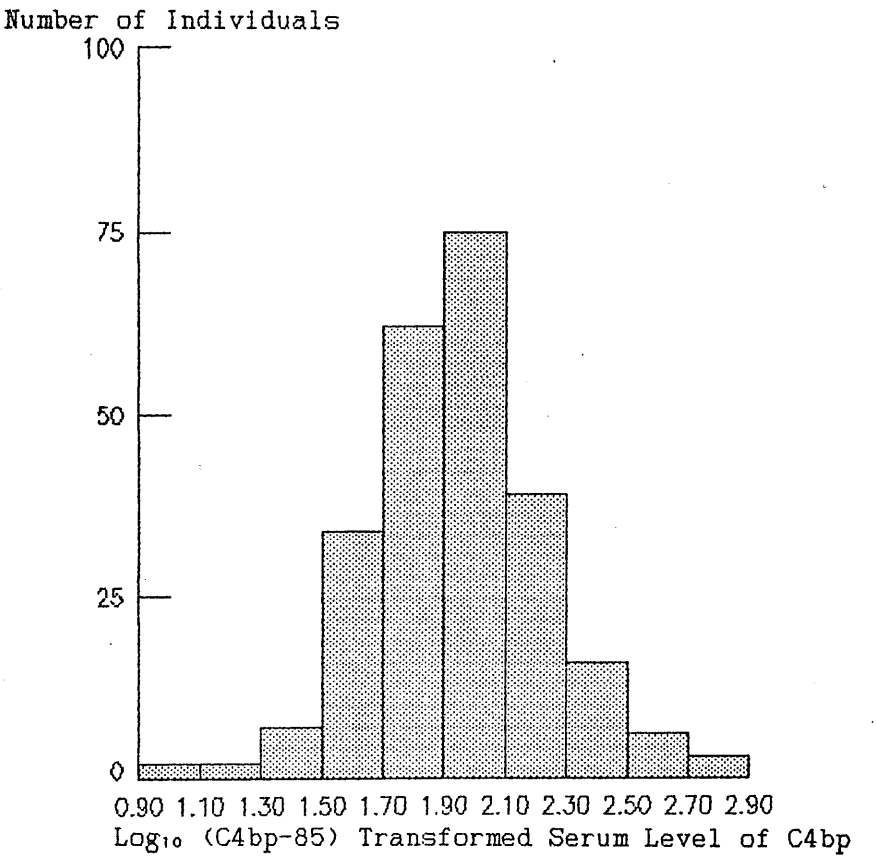
Legend

Frequency histogram showing the distribution of serum levels of C4bp in 123 pairs of twins after transformation of each to $log_{10}$ (C4bp-85).

(a)


Number of Individuals
125
100
75
50
25
0
75 125 175 225 275 325 375 425 475 525 575 625 675 725
Serum Level of C4bp (μg/ml)

(b)


Number of Individuals
100
75
50
25
0
0.90 1.10 1.30 1.50 1.70 1.90 2.10 2.30 2.50 2.70 2.90
Log10 (C4bp-85) Transformed Serum Level of C4bp

3.28 Effect of Age on C4bp Levels

There was a significant correlation between age and C4bp levels r=0.457 (Fig 3.9). It was calculated that for a C4bp concentration of 173 μg/ml that each years increase in age would, on average, result in an increase of about 1.6 μg/ml of C4bp (ie almost 1% per annum). The data were corrected for batch and age effect as described for CR1 assays and then analysed for differences between males and females.

3.29 Comparison of C4bp Levels in Males and Females

The mean value of C4bp levels in males was 173 μg/ml and that for females was 173 μg/ml. Thus it may be concluded that there is no difference at all between males and females in C4bp levels.

3.30 Genetic Analysis of C4bp Levels

The results of analysis of variance are shown in Table 3.6. The F ratio to compare dizygotic intra-pair variance (0.0455295) with monozygotic intra-pair variance (0.044597) was 1.0209 which is not significant (P > 0.1). Therefore there was no evidence or suggestion of genetic influence on the levels of C4bp.

3.31 Analysis of I Measurements in Twins

The distribution of I levels in all of the twins is shown in Fig 3.10(a). The distribution shows a strong positive skew and was transformed to a normal distribution by taking the $\log_{10}$ values of I measurements (Fig 3.10(b)). The geometric mean value of I levels was 55μg/ml. As individuals, not twins were assigned randomly to batches for assay of I no batch correction was necessary.

3.32 Effect of Age on I Levels

There was a significant correction between age and I levels (r=0.186; P < 0.005) (Fig 3.11). Each years increase in age was associated with an increase of about 0.00223 in $\text{Log}_{10}$ I levels. Therefore the I level increased by about 5% for every 10 years increase in age. After correction for age effect the $\text{Log}_{10}$ I levels were analysed for differences between the sexes.

3.33 Comparison of I Measurement in Males and Females

The mean values of I were calculated for males and females. For males there was a mean of 58μg/ml of I and for females a mean of 55μg/ml.There was no significant difference between I values in males and females as revealed by a pooled T test. Therefore it may be concluded that males and females have the same mean levels of I.

Fig 3.9 Correlation Between C4bp Levels and Age

Legend

Scatter graph showing a plot of serum concentration of $\log_{10}$(C4bp-85) against the age of the twin. The correlation coefficient is 0.457 which is significant.

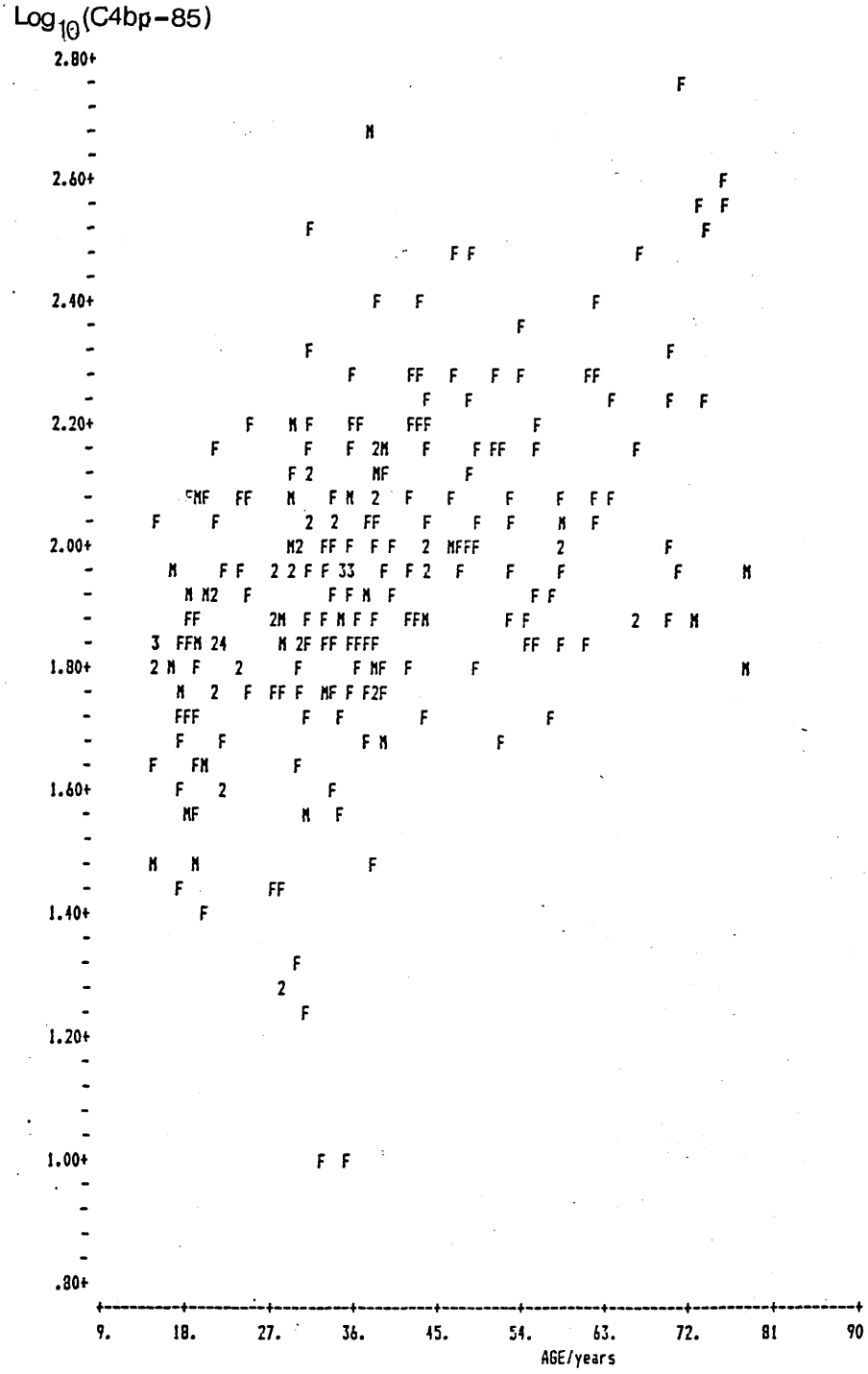

Log10(C4bp-85)
2.80+
2.60+
2.40+
2.20+
2.00+
1.80+
1.60+
1.40+
1.20+
1.00+
.80+
9.
18.
27.
36.
45.
54.
63.
72.
81
90
AGE/years

Table 3.6 Analysis of Variance of Log... (C4bp-85) Levels

| Source of Variance | Sum of Squares | DF | Mean Square | F-Ratio | Significance |
|---|---|---|---|---|---|
| Total | 20.3986 | 245 | 0.374195 | | |
| Batch Effect | 3.74195 | 10 | 3.47885 | 5.4336 | P < 0.001 |
| Age Effect | 3.47885 | 1 | 0.056315 | 50.5155 | P < 0.001 |
| Inter-pair | 7.64428 | 111 | 0.068867 | | |
| Intra-pair | 5.53349 | 123 | 0.0444988 | 1.5368 | P ~ 0.01 |
| ...divided as follows | | | | | |
| Monozygotic | 2.7204 | 61 | 0.044597 | 1.5442 | P < 0.05 |
| Dizygotic | 2.82283 | 62 | 0.0455295 | 1.5126 | P < 0.05 |
| ...subdivided as follows | | | | | |
| Dizygotic F | 1.918 | 35 | 0.0548 | 1.2567 | NS |
| Dizygotic M/F | 0.775587 | 21 | 0.0369327 | 1.8647 | NS |
| Dizygotic M | 0.129243 | 6 | 0.0215405 | 3.1971 | NS |
| Monozygotic F | 2.24031 | 53 | 0.04227 | 1.6292 | P ~ 0.025 |
| Monozygotic M | 0.480086 | 8 | 0.0600107 | 1.1476 | NS |

Fig 3.10(a) Distribution of I Levels

Legend

Frequency histogram showing the distribution of serum levels of I in 123 pairs of twins.

Fig 3.10(b) Distribution of $Log_{10}$ I Levels

Legend

Frequency histogram showing the distribution of serum levels of I in 123 pairs of twins after transformation of each to $log_{10}$ I.

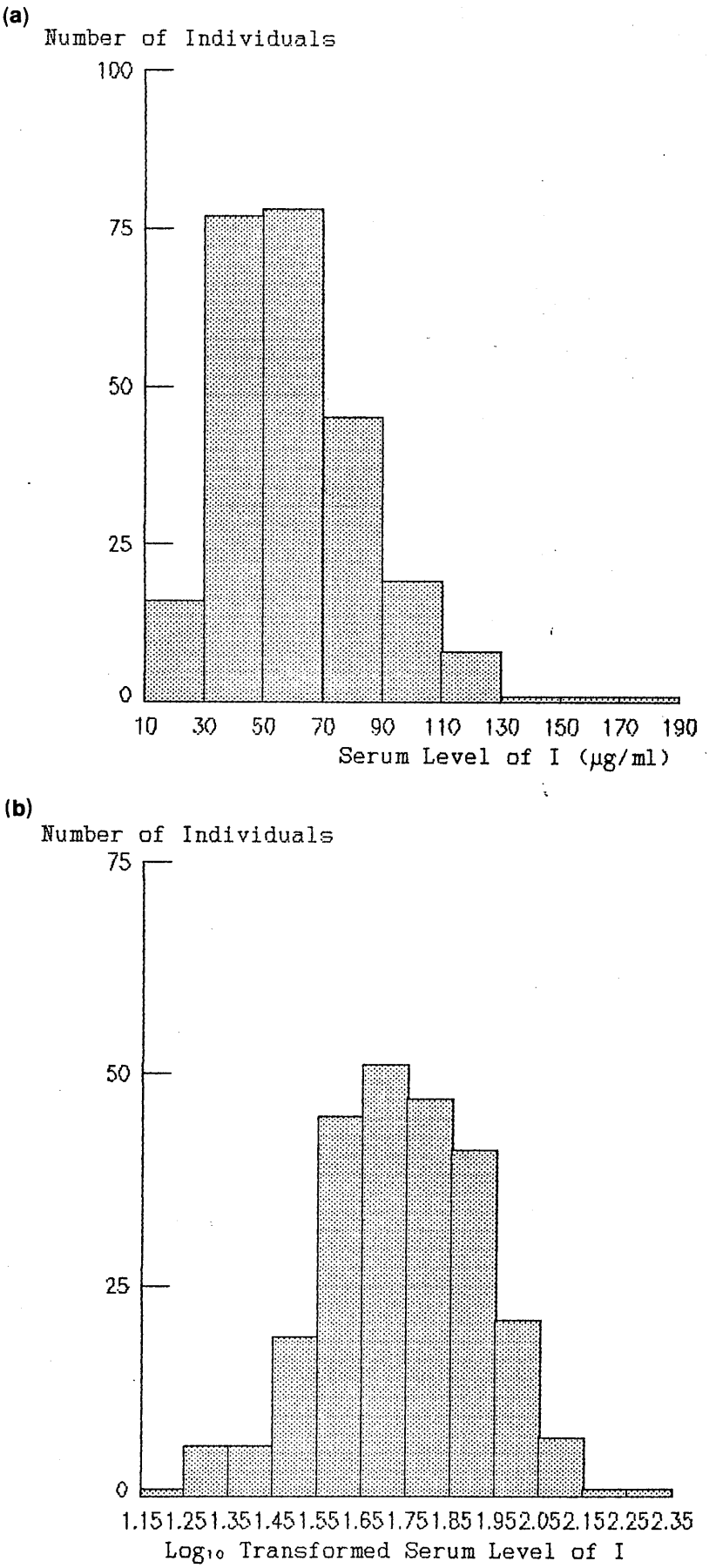
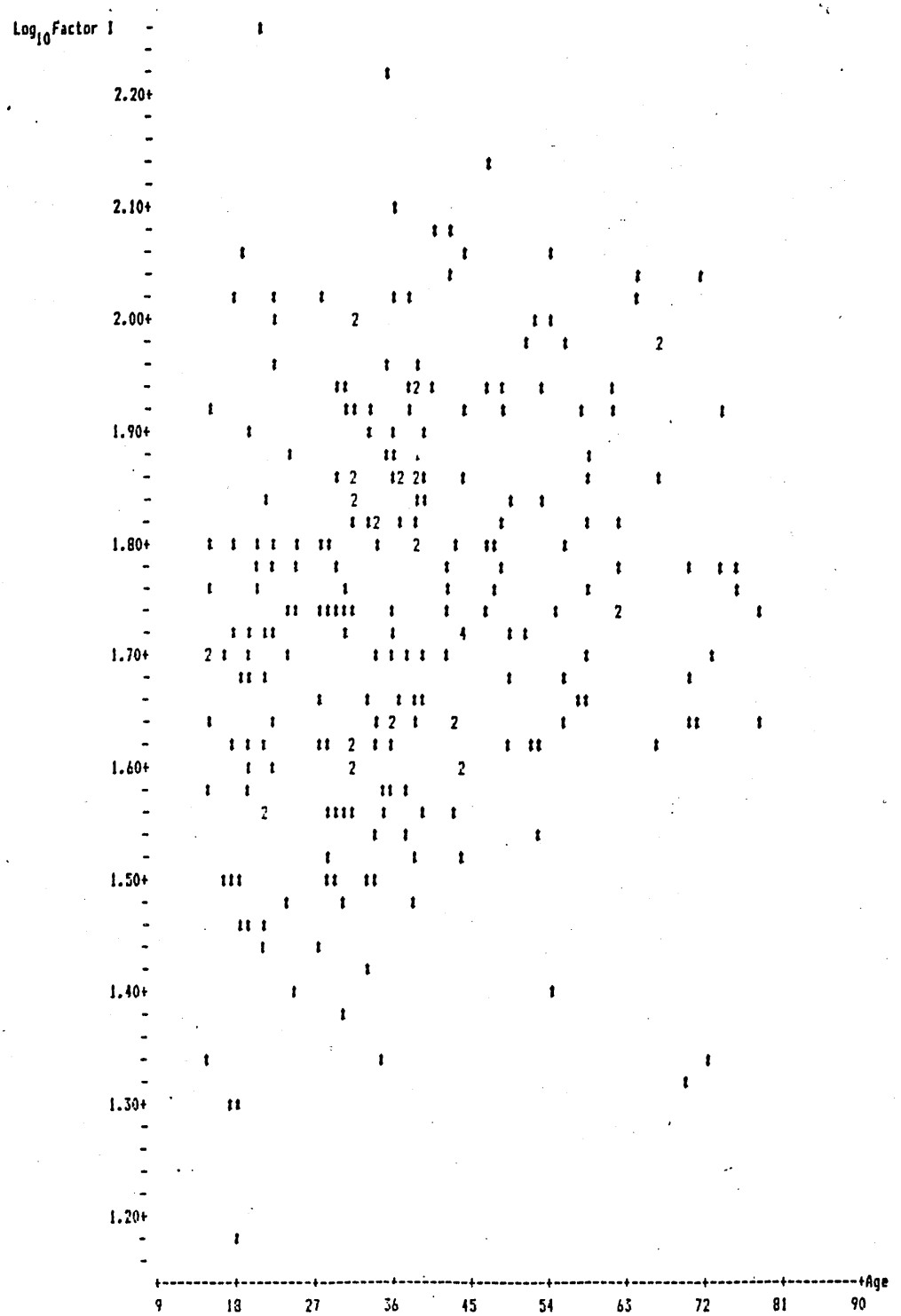

(a)
Number of Individuals
100
75
50
25
0
10 30 50 70 90 110 130 150 170 190
Serum Level of I (μg/ml)
(b)
Number of Individuals
75
50
25
0
1.15 1.25 1.35 1.45 1.55 1.65 1.75 1.85 1.95 2.05 2.15 2.25 2.35
Log10 Transformed Serum Level of I

Fig 3.11 Correlation Between I Level and Age

Legend

Scatter graph showing a plot of serum concentration of I against the age of the twin. The correlation is 0.186 which is significant.


Log10Factor I
2.20+
2.10+
2.00+
1.90+
1.80+
1.70+
1.60+
1.50+
1.40+
1.30+
1.20+
9
18
27
36
45
54
63
72
81
90
Age

3.34 Genetic Analysis of I Levels

The intra-pair and inter-pair variances and intra-class correlation coefficients were calculated for each set and subset. The results are shown in Table 3.7. They indicate that there is no real evidence of any correlation between twins of any category and therefore no evidence of any genetic influence on I levels.

3.35 Correlations Among C4bp, H, I and CR1

All the analysis for correlation between the proteins were carried out using assay results which had been corrected for batch and age, whether or not the batch and age effects were statistically significant. (Even if too small to be significant, various batch and age effects might in principle contribute cumulatively to an inter-protein correlation which would be significant but spurious).

The 4 protein levels of, C4bp, H, CR1 and I transformed and corrected for age and batch were then tested for male-female difference because, if present, the differences could violate the assumption of homogeneous data on which correlation analysis depends, and might give rise to spurious inter-protein correlations. As previously recorded there was no evidence of any difference between males and females and this was not changed by correction for batch and age effects.

Inter-protein correlations were then sought; these are recorded in Table 3.8(a) (b) and (c).

This was a preliminary screening analysis as for correlation analysis to be strictly valid, the individual items of data must be mutually independent. As the data come from sets of twins this requirement is not met, and the observed correlations may be rather more prone to extreme deviations from the true values than would be observed in a population of unrelated independent individuals.

However, the negative results from the analysis should be dependable, as the resemblance between twins can only exaggerate the random sampling error, not reduce it. Therefore it is reasonable to conclude that there is no correlation between CR1 and any of the other 3 proteins. Nor is there any significant difference in the correlation coefficients between the two sexes.

A further negative conclusion may be drawn concerning the correlation between I and C4bp. Since both of these factors correlate fairly well with H, one would expect that they would have to correlate with each other. Partial correlation analysis showed that the correlation between I and C4bp could be explained entirely by their correlation with H.

More formal rigorous analysis depended upon the division of each twin pair into 2 sets. Set 1 consisted of the member of each pair who had

Table 3.7 Analysis of Variance of $Log_{10}$ I Levels

| Set of Twins | Number of Pairs | Intra-pair Variance | Inter-pair Variance | r | P | Significance |
|---|---|---|---|---|---|---|
| Dizygotic F | 35 | 0,029882 | 0,025297 | -0,08309 | 0,63 | NS |
| Dizygotic M/F | 21 | 0,035302 | 0,0430768 | 0,09920 | 0,66 | NS |
| Dizygotic M | 6 | 0,009451 | 0,0279919 | 0,49518 | 0,26 | NS |
| Monozygotic F | 53 | 0,025089 | 0,039723 | 0,22579 | 0,1 | NS |
| Monozygotic M | 8 | 0,011929 | 0,0472148 | 0,59661 | 0,09 | NS |
| Dizygotic | 62 | 0,029741 | 0,032557 | 0,0452 | 0,72 | NS |
| Monozygotic | 61 | 0,023363 | 0,0399456 | 0,26194 | 0,04 | ? |
| All Twins | 123 | 0,026578 | 0,0373 | 0,16785 | 0,062 | NS |

Notes

r = intra-class correlation coefficient.

Table 3.8 Inter-Protein Correlation Coefficients

(a) Whole Data Set

| | C4bp (r) | H (r) | CR1 (r) |
|---|---|---|---|
| H | 0.427 ($P < 10^{-6}$) | | |
| CR1 | 0.066 (NS) | 0.074 (NS) | |
| I | 0.188 ($P < 0.005$) | 0.248 ($P < 10^{-4}$) | 0.048 (NS) |

(b) Females Only

| | C4bp (r) | H (r) | CR1 (r) |
|---|---|---|---|
| H | 0.447 ($P < 10^{-6}$) | | |
| CR1 | 0.028 (NS) | 0.049 (NS) | |
| I | 0.17 ($P < 0.02$) | 0.243 ($P < 10^{-3}$) | 0.006 (NS) |

(c) Males Only

| | C4bp (r) | H (r) | CR1 (r) |
|---|---|---|---|
| H | 0.342 ($P < 0.02$) | | |
| CR1 | 0.241 (NS) | 0.210 (NS) | |
| I | 0.287 ($P < 0.05$) | 0.247 (NS) | 0.257 (NS) |

the lower serum serial number while Set 2 consisted of the member of each pair who had the higher serum serial number. Studying set 1 alone for evidence of inter-protein correlation was completely valid and set 2 was also studied for confirmation. When analysis of the 2 twin sets were performed separately, as expected, there was no significant difference between the P values obtained for the inter-proteins correlations. Therefore the evidence from twin 1 and twin 2 was combined to obtain a combined confidence level, or P value. These P values are shown in Table 3.9. Fig 3.12(a) and (b) illustrate correlations between C4bp and H and between I and H respectively.

In addition to calculating the inter-protein correlation coefficients for twin 1 and twin 2, the inter-protein correlation coefficients were also calculated for the differences between twins ie twin 1 - twin 2, and for the sums of twin 1 + twin 2. This further analysis was undertaken in an attempt to determine if the factors responsible for the correlations between the different proteins occured as a result of genetic or environmental influences. If the correlation was a result of genetic factors then the correlation would be more apparent in the sum and not the difference between twins. This occurs when each individual in the twin pair has the same amount of a particular protein. The subtraction of one from the other cancels out the genetic influence, and what remains must be due to the environmental effect. Conversely if the serum levels of 2 proteins correlated because they were both influenced by the same environmental factors, then the correlation of the differences between individual twins would be stronger than the correlation of the sum of the values of each set of twins.

The correlation based on the sum of pairs of assays ie twin 1 + twin 2 are listed in Table 3.10 and those based on the differences between pairs are listed in Table 3.11.

The results of these studies show that the correlation between H and C4bp and between C4bp and I are rather more apparent in the differences within twin pairs than in the sums of twin pairs. This might imply that environmental influences were predominant in determining the correlations. However, this conclusion did not satisfy any formal significance tests. On the other hand the correlation between H and I was marginally more apparent in the sums of twin pairs than in the differences. This might indicate a genetic influence on the inter-protein correlation but again this conclusion was not supported by any formal statistical tests.

In summary the main conclusions which can be drawn from this correlation analysis are (i) CR1 numbers did not correlate with C4bp, H or I, (ii) H levels correlated significantly with C4bp ($r=0.427$; $P < 0.00001$), (iii) H levels correlated significantly with I ($r=0.248$; $P < 0.0001$), (iv) C4bp and I correlated with each other ($r=0.188$; $P < 0.01$) but this is no more than would be expected from conclusions (ii) and (iii), (v) there is no evidence of any male-female differences in the mean values of any of the

Table 3.9 Inter-Protein Correlation Coefficients for the Combined Twin 1 + Twin 2 P Values

| | C4bp (r) | H (r) | CR1 (r) |
|---|---|---|---|
| H | 0.427 ($p < 10^{-5}$) | | |
| CR1 | 0.066 (NS) | 0.074 (NS) | |
| I | 0.188 ($P < 0.01$) | 0.248 ($P < 10^{-4}$) | 0.003 (NS) |

Table 3.10 Inter-Protein Correlation Coefficients for Twin 1 + Twin 2

| | C4bp (r) | H (r) | CR1 (r) |
|---|---|---|---|
| H | 0.339 ($p < 0.001$) | | |
| CR1 | 0.008 (NS) | -0.020 (NS) | |
| I | 0.114 (NS) | 0.280 ($P < 0.002$) | -0.063 (NS) |

Table 3.11 Inter-Protein Correlation Coefficients for Twin 1 - Twin 2

| | C4bp (r) | H (r) | CR1 (r) |
|---|---|---|---|
| H | 0.552 ($P < 10^{-6}$) | | |
| CR1 | 0.125 (NS) | 0.169 (NS) | |
| I | 0.290 ($p < 0.001$) | 0.205 ($P < 0.005$) | (NS) |

Fig 3.12(a) Correlation Between C4bp and H

Legend

Scatter graph showing serum levels of C4bp plotted against serum levels of H. There is a significant correlation ($r = 0.427$).

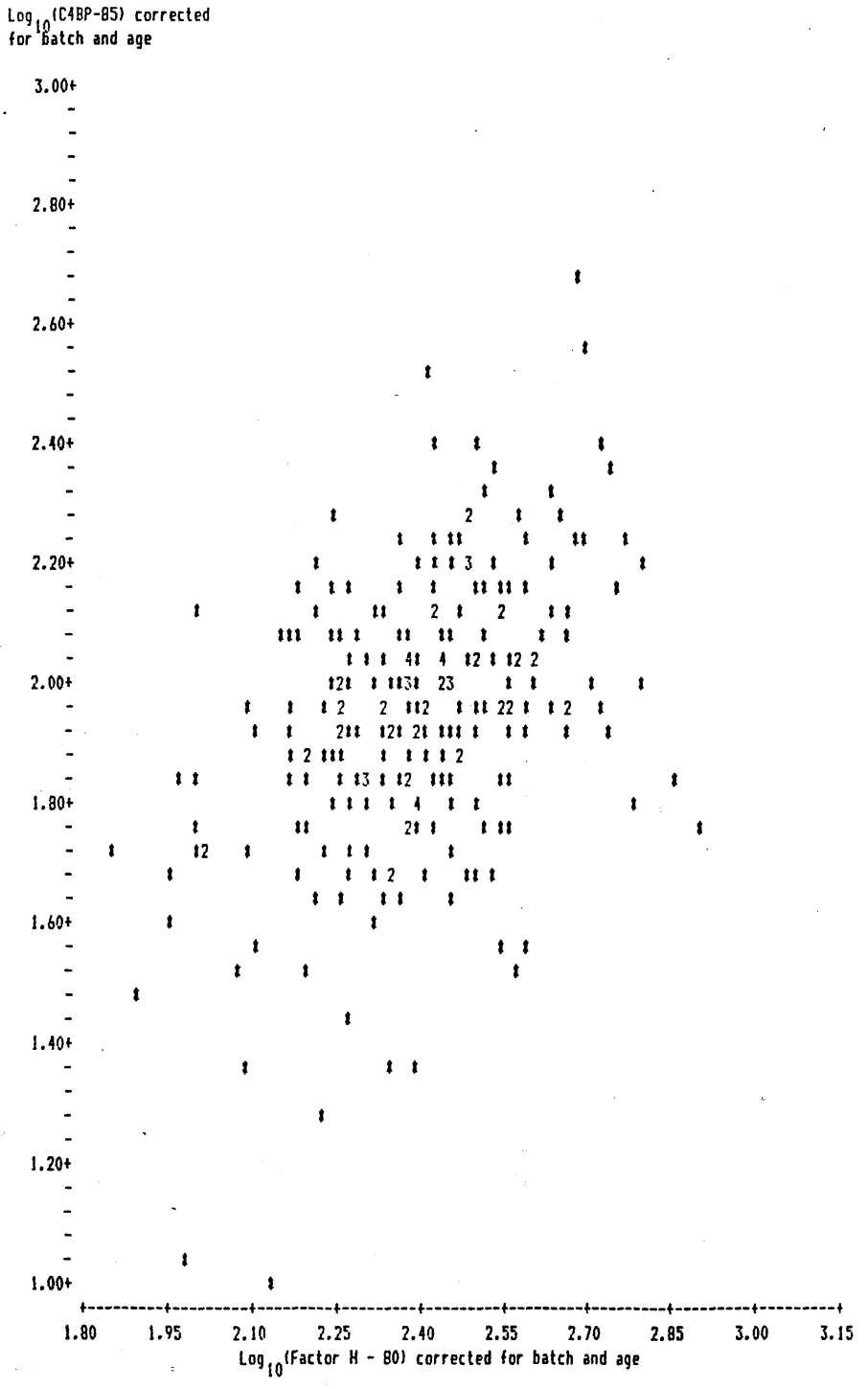
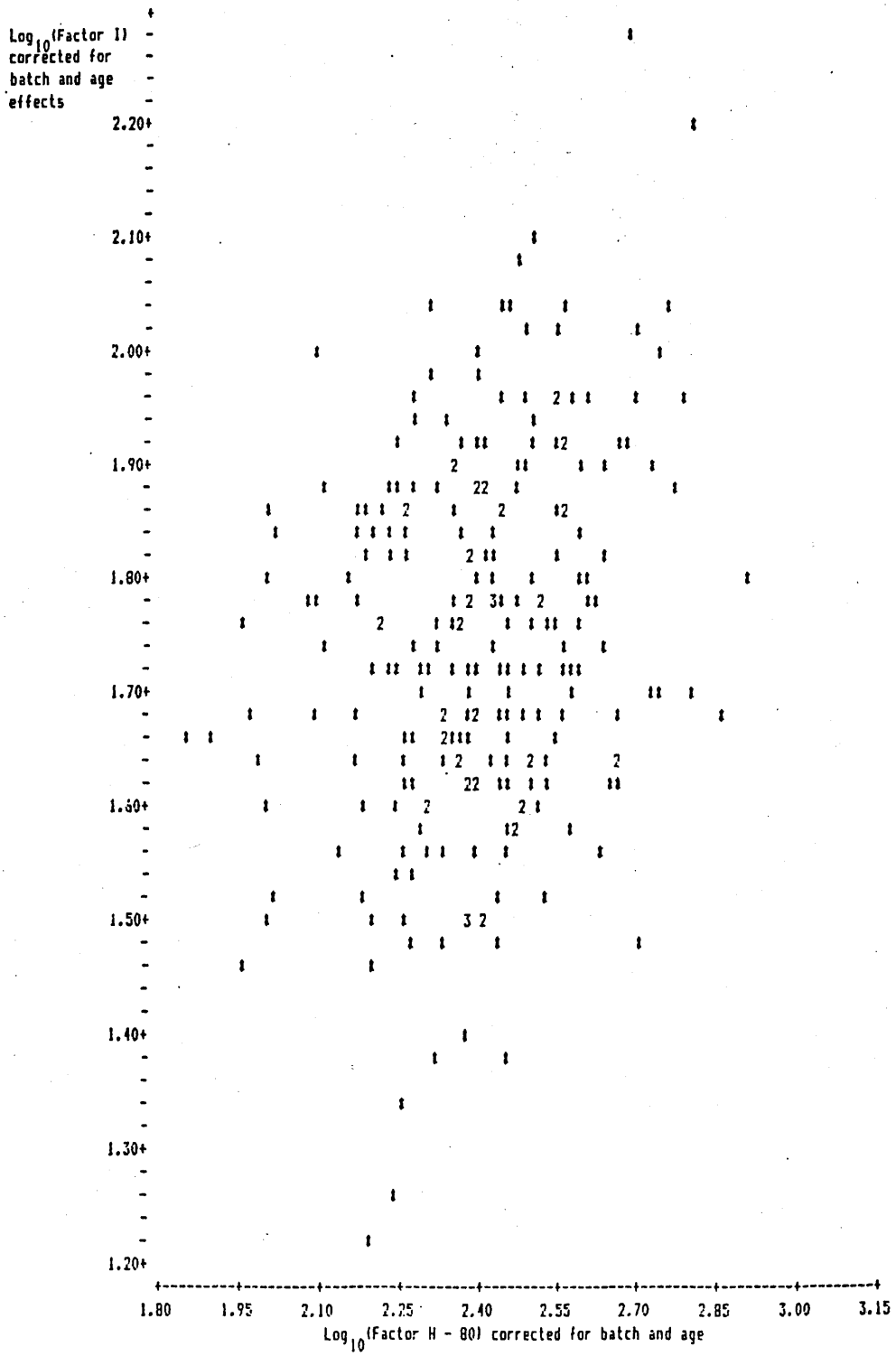
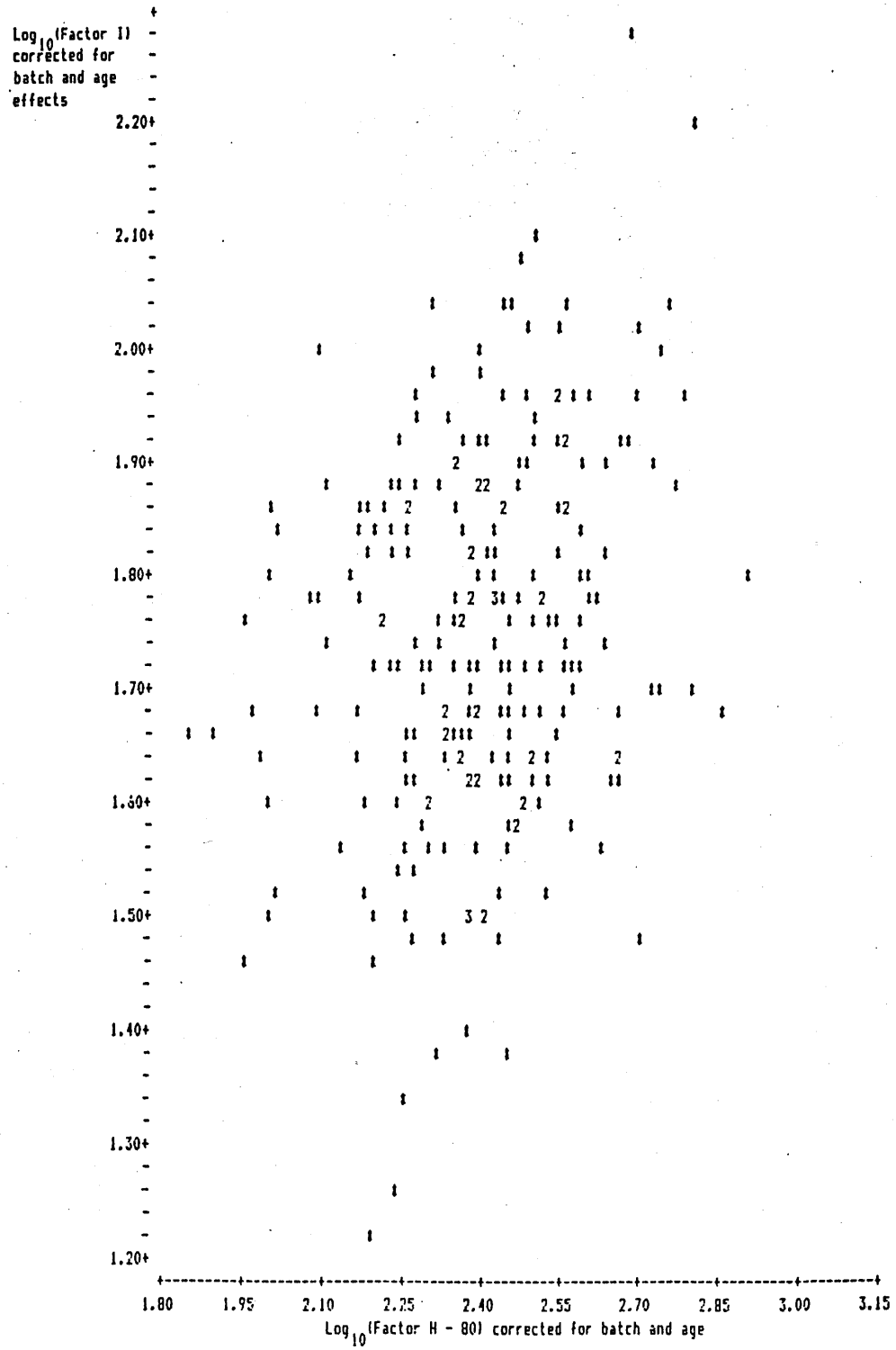

Log10(C4BP-85) corrected for batch and age
3.00+
2.80+
2.60+
2.40+
2.20+
2.00+
1.80+
1.60+
1.40+
1.20+
1.00+
1.80
1.95
2.10
2.25
2.40
2.55
2.70
2.85
3.00
3.15
Log10(Factor H - 80) corrected for batch and age

Fig 3.12(b) Correlation Between I and H

Legend

Scatter graph showing serum levels of I plotted against serum levels of H. There is a significant correlation ($r = 0.248$).


Log10(Factor I) corrected for batch and age effects
2.20+
2.10+
2.00+
1.90+
1.80+
1.70+
1.60+
1.50+
1.40+
1.30+
1.20+
1.80
1.95
2.10
2.25
2.40
2.55
2.70
2.85
3.00
3.15
Log10(Factor H - 80) corrected for batch and age


Log10(Factor II) corrected for batch and age effects
2.20+
2.10+
2.00+
1.90+
1.80+
1.70+
1.60+
1.50+
1.40+
1.30+
1.20+
1.80
1.95
2.10
2.25
2.40
2.55
2.70
2.85
3.00
3.15
Log10(Factor H - 80) corrected for batch and age

4 proteins and finally (vi) C4bp, H and I all increased significantly with age.

## 3.36 Discussion

Mendel was the first to explain heredity simply in terms of genes. His experiments with pea varieties that differed in qualitative clear-cut visible traits such as size and colour showed that some differences were simply inherited and caused by one pair of alleles at a single gene locus. However, not all inheritance is so clear-cut: variations such as those in stature, weight and skin colour in humans are quantitative rather than qualitative and can be accounted for by supposing that continuously varying traits are due to the joint action of several or many genes each of which has individually only a small effect on the traits in question. Such inheritance is said to be under polygenic control. Polygenes, the genes that bring about heritable variations in quantitative traits are genes, the alleles of which, produce small phenotypic differences, which are similar to differences which could be caused simply by environmental factors.

The number of CR1 sites per E have been shown to vary by as much as 10-fold among normal healthy individuals as assessed functionally by binding of dimeric C3b as well as antigenically using polyclonal or monoclonal antibodies (Wilson et al 1982; Iida et al 1982; Minota et al 1984; Ross et al 1985; Walport et al 1985a; Holme et al 1986). A genetic basis for this variation has been proposed.

Wilson et al (1982) enumerated CR1 on E from a section of the normal population and noted that the numbers appeared to fall into a trimodal distribution. They suggested that the difference in CR1 numbers was clear-cut with individuals having 2 codominant alleles coding for either low or high CR1 expression. This model was used to explain why patients with SLE had reduced E CR1 levels as it was concluded that these patients had an increased prevalence of the allele determining low numerical expression of E CR1. In agreement with this model of inheritance, Nojima et al (1985) found that the capacity of E to bind to radiolabelled aggregated human γ globulin ($^{125}$I-AHG) was distributed in a trimodal fashion in the normal population. Thirteen percent of normals showed low binding capacity, 58% intermediate and 29% high. These frequencies did not deviate significantly from frequencies calculated from the Hardy-Weinberg equilibrium assuming a 2 codominant allele model determining low and high binding values. Others have disagreed with this simple model of inheritance suggesting that inheritance is as a result of the contribution of many genes (Walport et al 1985a). This proposal is based on the observation that E CR1 numbers in the normal population are distributed continuously and not in clearly defined groups of individuals with high, intermediate or low CR1 numbers (Ross et al 1985; Walport et al 1985a; Holme et al 1986). Normally distributed quantities are usually the result of a number of factors both genetic and environmental; however the distribution by itself gives no indication as to how many of these factors are environmental and how many are genetic. Walport et al (1985a) studied CR1 numbers on 86 normal subjects. The range of CR1 numbers was large (145-1214 sites/E) and they were distributed in a normal fashion

with no evidence of a trimodal grouping. In an attempt to study the genetic influence on this distribution the mean parental CR1 numbers for 10 normal families were plotted against the CR1 numbers of each of their children . A strong correlation ($r=0.58$, $P < 0.001$) was found between CR1 numbers in parents and children. They stated that although their data was insufficient to establish the precise nature of inheritance which may be controlled by one or more genes, their data fitted a polygenic model of inheritance (ie influenced by many genes) analogous to that involved in the control of height. However upon closer analysis of their data it would appear that some fundamental errors were made in the calculation of their correlation coefficient between parents and children. Firstly their data were not homogeneous in that a mixed population of normal and SLE families were included in the calculation; secondly for a valid correlation all data must be independent; this was not the case as more than one child from each family was included and thirdly they did not state whether grouping of samples into batches for E CR1 assays were random. A spurious correlation could have arisen simply because all samples from the one family were measured in the same batch. For a valid correlation the data must be homogeneous, independent and necessary corrections must be made for batch effects when required. As this correlation was presented as their strongest evidence for an inherited component in the expression of E CR1 their conclusions must be viewed with caution.

In order to study the proposed genetic inheritance of CR1 in the normal population it was decided to study E CR1 levels in monozygotic and dizygotic twins. This study involved the collection of blood samples from 123 pairs of twins. It was necessary to freeze the blood as CR1 sites could not be measured at the time of venous blood sampling. Thawing of blood samples resulted in a variable degree of lysis. However as can be seen from Table 3.3 when the number of E which had lysed were accounted for the final E CR1 number was within the inter-assay error for a fresh unfrozen sample.

CR1 levels in the twins were distributed in a log-normal fashion similar to that observed by Holme et al (1986). This distribution was transformed to a normal bell shaped distribution by converting each measurement to the $\log_{10}$. The frequency histogram obtained by this transformation (Fig 3.4(b)) showed that the transformed data approximated to a normal distribution. There was no evidence of distinct groups of individuals having high or low phenotypes. Thus the distribution was not consistent with a simple model of genetic inheritance. The geometric mean value of CR1 sites/E obtained for all the twins studied was 837. This is within the normal range of 300-1200 sites/E reported by Hogg et al (1984) who used the same monoclonal antibody to quantify CR1 sites on the E of 38 normal subjects.

Using the $\log_{10}$ transformed E CR1 numbers statistical analyses were performed to determine if there were significant differences between the sexes or if there were differences associated with age. No significant

differences were found between CR1 number in males or females and there were no significant correlations between CR1 and age. The results are in agreement with reports from various other groups. Iida et al (1982) found that there was no significant difference in CR1 number according to age, sex or blood type. Jouvin et al (1986) found that CR1 number was independent of age and sex of the donors and Wilson et al (1982) found no significant difference in CR1 numbers between males and females.

The data were then analysed to determine how much the distribution was dependent on genetic factors . The basis for the genetic analysis of CR1 levels on the E of identical and nonidentical twins was the comparison of differences in CR1 levels within sets of twins (intra-pair variance) and between sets of twins (inter-pair variance). If it is to be supposed that CR1 numbers are inherited then the numbers of CR1 sites on the E from identical twins should be closer than the number of CR1 sites on the E from nonidentical twins. This in turn implies that the intra-pair variation in identical twins should be smaller than the inter-pair variation between all twins if CR1 numbers are genetically controlled. This however was not found to be the case as there was no resemblance between twins of any of the sets or subsets as seen by the fact that the intra-pair variances were not any smaller than the inter-pair variances (Table 3.4) The F ratio to compare monozygotic intra-pair variance with dizygotic intra-pair variance was not significant which indicates that the difference between monozygotic twins was not any smaller than the difference between dizygotic twins.

From this genetic analysis it was concluded that the data failed to demonstrate any genetic influence on E CR1 levels in normal twins. It must therefore be supposed that differences in E CR1 levels must arise largely or wholly as a result of environmental factors. In support of this conclusion experiments in our own laboratory by Dr E Holme have shown that E CR1 levels on the cord blood of new born monozygotic twins differ quite markedly from each other.

A recent publication which would seem to contradict this conclusion is that by Wilson et al (1986a) which states that there appears to be a restriction fragment length polymorphisim involving fragments of 7.4kb and 6.9kb which correlates with variation in the quantitative expression of E CR1. In a preliminary study they showed that 4 individuals with high CR1 levels had only the 7.4kb fragment, 4 individuals with intermediate CR1 levels had both the 7.4kb and the 6.9kb fragments and 4 individuals with only the 6.9kb fragment had low CR1 numbers. However even in this small sample there were exceptions. Two individuals with intermediate expression had only the 6.9kb fragment. A larger study of 50 normal individuals showed that E CR1 numbers in groups with the 7.4kb fragment overlapped the group with the 7.4kb and 6.9kb fragments, while the group with the 6.9kb fragment had low CR1 numbers. These data would appear to suggest that while low E CR1 numbers are genetically controlled the phenotype of those with the restriction fragment length polymorphism associated with high CR1 numbers is modified by factors which are not

the product of the CR1 structural gene. These results suggest to me that it is not possible to make clearly defined groupings on the basis of possession of one or other or both of these fragments.

As mentioned in the introduction to this chapter CR1, C4bp and H are thought to comprise a new human complement regulatory gene family (Holers et al 1986). They each have similar roles in that they all interact with either C4b or C3b. In addition they all act as cofactors for either the I mediated cleavage of C3b or C4b. There may also be structural similarities in that they all might possess a homologous domain involved in C3b/C4b binding (Sim 1985; Sim et al 1986). Sequence studies on C4bp have indicated that it is composed of 8 internally homologous repeating segments about 60 amino acids long. H is made up of 20 of the same repeating structures and CR1 is thought to contain at least 8 of these repeating units (Sim et al 1986).

Gene linkage studies by Rodriguez de Cobra et al (1985) have shown that the structural genes for each of the proteins are closely linked. Because of the close association between these 3 proteins it was decided to study C4bp and H levels in the serum collected from the twins to see if the levels of these proteins correlated with levels of CR1 and also to investigate the influence of genetic factors on C4bp and H. In addition, I, the enzyme for which all these proteins are cofactors, but which itself is not part of this complement family, was studied as a control.

It was found that like CR1 there was no difference in levels of H and C4bp between males and females. However, unlike CR1 there was a significant age effect on levels of H and C4bp, both of which increased with age (Fig 3.7 and Fig 3.9). Like CR1 there did not appear to be any evidence of a genetic influence on H or C4bp levels (Tables 3.5 and Table 3.6). There were no correlations found between CR1 levels and C4bp or H levels although there was a significant correlation between H and C4bp levels (Fig 3.12(a)).

Thus it would appear that there are differences between the regulation of CR1 levels and the regulation of the levels of the other 2 cofactor proteins. This may in part be accounted for by the fact that H and C4bp are plasma proteins whereas CR1 is a membrane-bound protein. In addition CR1 has a number of unique roles which are not possessed by the other cofactor proteins. It is thought to act as a cofactor for the I mediated breakdown of surface-bound iC3b to C3dg and C3c (Medof et al 1982). It is also involved in phagocytosis and the accompaning metabolic events (Pommier et al 1984; Changelian and Fearon 1985). It may play a role in the regulation of immunoglobulin secretion from lymphocytes (Daha et al 1984) and in the retention of antigens in lymphoid follicles (Reynes et al 1985) and <u>in vivo</u> it has been shown that E CR1 is involved in the transportation of immune complexes (Medof and Oger 1982; Cornacoff et al 1983).

Levels of E CR1 are significantly reduced in patients with active SLE (Miyakawa et al 1981; Ross et al 1985; Holme et al 1986) and so are levels of C4bp and H (Whaley, Schur and Ruddy 1979; Daha et al 1983). The cause of the reduced levels of 3 proteins which control complement turnover is not known. However the environmental factors which result in the reduction of E CR1 in active SLE may be different to those which result in the reduction of H and C4bp levels as in normals expression of CR1 and H or C4bp do not correlate.

Analysis of I revealed that there was no significant difference in serum concentration of I between males and females. There was a significant increase in I levels with age (Fig 3.11)($r=0.186$; $P < 0.005$). Increase in I, C4bp and H levels with age may be as a response to an increasing requirement for the regulation of complement activation.

There was no evidence for any genetic influence on I levels (Table 3.7). I levels correlated significantly with H and C4bp levels but not with CR1. Correlations between I and C4bp and H may be of biological value as I requires the presence of one of its cofactors to mediate cleavage of C3b and C4b. Correlations between H and I have previously been noted by Whaley et al (1979).

Thus it may be concluded from this study that environmental factors and not genetic factors are mainly or wholly responsible for variations in the expression of I, C4bp, H and CR1. The environmental factors which influence CR1 expression appear to be different to those governing the expression of the other 3 proteins. The precise nature of the factors governing expression of all of these proteins is not known. However in the following chapter the results of investigations into some of the environmental factors which may influence CR1 expression are reported.

# CHAPTER FOUR

## 4.1 Introduction

The results from the previous chapter demonstrated that E CR1 number could be measured by monoclonal anti-CR1. By this method E CR1 numbers were analysed on 122 sets of twins. This showed that the major factors governing CR1 expression are environmental and not genetic.

Patients with SLE have reduced numbers of E CR1 (Miyakawa et al 1981; Wilson et al 1982; Walport et al 1985a; Holme et al 1986). Recent experimental evidence suggests CR1 numbers are reduced as a consequence of the disease (Ross et al 1985; Walport et al 1985a; Holme et al 1986). If it is to be concluded that these numbers are not reduced by genetic factors but are acquired as a consequence of the disease, mechanisms must exist which modulate CR1 numbers.

One possibility may be that the E of SLE patients do not synthesise sufficient quantities of the receptor. Two main pieces of evidence exist to refute this possibility. Firstly, it was found that transfusion of E with high CR1 numbers into SLE patients expressing low CR1 numbers resulted in a gradual loss of CR1 from the transfused cells. There was a loss of 50% of CR1 during the first 5 days after transfusion (Walport et al 1985b). Secondly, E normally circulate in the bloodstream for over 3 months before they are destroyed, however, serial studies on SLE patients have found that their CR1 numbers change by over 100% in 2 months or less (Ross et al 1985; Holme et al 1986). This suggests that some mechanism operates in these patients to remove or block E CR1.

It has been reported that CR1 levels per E are higher on younger less dense E in both normal and patient groups than on older E (Wilson et al 1982; Sim, Roord and Sim 1983; Ripoche and Sim 1986). Thus there exists *in vivo* a general nonpathological mechanism for causing a reduction in E CR1 numbers. Ripoche and Sim (1986) have suggested that this same mechanism may also be responsible for the accelerated loss of E CR1 which is seen in SLE. They consider the most likely mechanism to be the removal of CR1 by proteolytic enzymes.

CR1 is known to be destroyed by proteases. Nelson and Nelson (1959) originally reported that as immune adherence was trypsin sensitive, this could indicate the destruction of a receptor responsible for the adherence. Four years later Nelson (1963) found that adherence was also destroyed by chymotrypsin and papain. As discovered earlier in attempts to purify CR1 (Chapter 2), the purified receptor is very sensitive to proteolysis. This sensitivity of isolated CR1 to proteolysis was also observed by Sim (1985). Ripoche and Sim (1986) have since found that CR1 is sensitive to the plasma proteases, thrombin and plasmin and conclude that it is possible that proteolysis of CR1 occurs when E carrying immune complexes (Siegel et al 1981; Cornacoff et al 1983; Medof & Oger 1982) come into contact with the proteinase-rich tissue phagocytes of the reticulo-endothelial system in liver and spleen. Such a mechanism of removal was also suggested by Ross et al (1985). Removal of CR1 in this

manner may occur under normal physiological conditions but in SLE as the immune complex load is greater (Tan et al 1982) there would be an increased chance of CR1 removal.

Alternative explanations for the low E CR1 numbers in SLE patients include, (i) the internalisation of CR1 by E. This is a remote possibility as there is no electron microscopic evidence for the existence of endocytic vesicles in E (Dr I More, personal communication) and (ii) the blockade of CR1 by antigen/antibody complexes which may hinder access of the radiolabelled antibody to the receptor. Studies in our own department by Dr E Holme suggest that this may be the case. However, the situation appears to be complex as illustrated by the observation that when E from SLE patients were examined for the presence of immune complex material such as C3 antigens or IgG antigens, there was no correlation between IgG content and CR1 level but some correlation between C3 levels and CR1 numbers (Dr E Holme, personal communication). Thus it is possible that the receptors are not being blocked by immune complexes but by some other as yet unknown factor such as polysaccarides or histone complexes which activate complement (Robey, Jones and Steinberg 1985).

In this chapter I have investigated some of the more likely possibilities which might cause an alteration in CR1 expression. Firstly, the sensitivity of CR1 to enzymes was studied. The serum proteases, plasmin, thrombin and kallikrein, the leukocyte proteases elastase and cathepsins C and D and the pancreatic enzyme trypsin were examined for their ability to remove CR1 from E. This was achieved by measuring E CR1 numbers by RIA before and after treatment with the enzymes. In addition the sensitivity to trypsin was assessed by immunoprecipitation of CR1 from surface labelled E before and after trypsin treatment.

The possibility that an as yet unknown factor exists in SLE sera which can remove or block CR1 was investigated by incubating E overnight in SLE sera followed by analysis of CR1 by RIA and surface labelling.

A preliminary study was also undertaken to determine if blockade of CR1 resulted from incubation of opsonised aggregated immune complexes with E. In addition studies on the modulation of CR1 expression in response to oral antigen (cows milk) challenge were performed.

Materials and Methods

4.2 Chemicals and Reagents

Antisera were obtained from the following sources:

Dakopatts, Mercia Brocades Ltd, Brocades House, Pyrford Rd, Weybridge, Surrey.

Mouse Monoclonal Anti-CR1

Miles Scientific, Stoke Court, Stoke Poges, Slough

Rabbit Anti-Mouse IgG (H+L fractions)

Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Scotland

Sheep Anti-Human 1gG

Cappel laboratories, Cochranville, PA 19330 USA

Sheep Anti-Rabbit $F(ab')_2$ Fragment
Sheep Anti-Rabbit IgG

University Animal Laboratory, Western Infirmary, Glasgow

Mouse Serum

The following reagents were purchased from these companies:

Sigma Chemical Company, Fancy Rd, Poole, Dorset

Aprotonin
Cathepsin C (from bovine spleen)
Cathepsin D (from bovine spleen)
Elastase (from porcine pancreas)
Glucose Oxidase
Iodoacetamide
Kallikrein (from human plasma)
Lactoperoxidase (from bovine milk)
Pepsin
Plasmin (from human plasma)
Protein A Crude Cell Suspension (10% w/v) of S. Aureus
Soybean Trypsin Inhibitor (SBTI)
Trypsin (from bovine pancreas)
Thrombin (from human plasma)

BDH Chemical Company Ltd. Poole. Dorset

Potassium Iodide
Sodium Acetate

Kodak Ltd. Dallimore Rd. Manchester

X-Ray Developer LX24
X-Ray Liquid FixerFX40
X-AR5 X-Ray Film

Whatman. Laboratory Sales Ltd. Unit 1. Coldred Rd. Maidstone. Kent

DEAE52 Diethylaminoethyl Cellulose (DE52 Cellulose)

Pharmacia. Pharmacia House. Midsummer Boulevard. Central Milton Keynes. Bucks

Sephadex G-150

4.3 SDS-PAGE in Glass Tubes

Buffers

Tank buffer

100mls of sodium phosphate buffer (1 mol $l^{-1}$ pH 6.5) and 10mls of 10% (w/v) SDS were made up to 1 litre with deionised water.

Gel Recipe (7%)

10mls of acrylamide/bisacrylamide solution, prepared as described in chapter 2.6(h), 25.6mls of deionised water, 4mls of phosphate buffer (1 mol $l^{-1}$), 10mls of 10%(w/v) SDS, 20µl of TEMED and 40mg of ammonium persulphate were mixed together, the last ingredient being added immediately prior to pouring the gel.

Destain Solution and Stain Solution

These solutions were prepared as described in chapter 2.6(h).

Sample Buffer

Sample buffer was prepared as described in chapter 2.6(h).

Procedure

The lower ends of glass gel tubes were sealed with Parafilm and placed into a rack. The gel mixture (7½%) was added to the tubes leaving a space of 1cm at the top. The gel was overlaid with water and left to

polymerize at room temperature for 1 hour. The Parafilm was removed and the tubes were placed into an electrophoresis tank filled with tank buffer. An equal volume of sample and sample buffer were mixed and applied to each gel after removal of the water. The electrophoresis was run at a constant current of 6mA/tube until the bromophenol blue marker was almost at the end of the gel. The gels were then removed from the tubes and stained with coomassie brilliant blue before being destained and photographed.

4.4 Immunoelectrophoresis

Buffers

Tank Buffer

17g of sodium barbitone and 23.5mls of hydrogen chloride (1 mol $l^{-1}$) were made up to 2 litres with deionised water to give a buffer with a pH of 8.4.

0.5%(w/v) Bromophenol Blue

0.5g of bromophenol blue were dissolved in 100mls of deionised water.

Reagents

Agarose Gel was prepared as described for double immunodiffusion in chapter 2.6(f).

Procedure

A glass plate (8x8cm) which had been washed in hot water and swabbed with alcohol was coated with 10mls of agarose gel. Wells and troughs were cut using a template and the wells were filled to the brim with sample using a micropipette. One well was filled with normal human serum containing 0.5%(w/v) bromophenol blue which acted as a marker allowing the progress of the separation to be followed. The gel was placed into an electrophoresis tank, filled with tank buffer, in direct contact with the wicks and electrophoresed at a constant current of 1.5mA/cm of gel until the bromophenol blue marker was 1cm from the end of the plate. When the electrophoresis was complete the troughs were filled with an appropriate antiserum and left at room temperature in a humid chamber for precipitin arcs to develop.

## 4.5 Preparation of $F(ab')_2$ anti-CR1 and $F(ab')_2$ Normal Rabbit Serum (NRS)

### Buffers

### Phosphate Buffer

Phosphate buffer (0.01 mol $l^{-1}$ pH 7.6) was prepared as described in chapter 2.8.

### PBS

PBS was prepared as described in chapter 2.6(c).

### Sodium Acetate

Sodium acetate was prepared as a 0.1 mol $l^{-1}$ solution at pH 4.5.

### Chromatograhpic Material

### DEAE 52 Cellulose (DE52)

DE52 was equilibrated in phosphate buffer (0.01 mol $l^{-1}$ pH 7.6) and poured into a 60ml syringe barrel.

### Sephadex G-150

Sephadex G-150 was equilibrated in PBS degassed and poured into a 1.5x90cm column.

### 4.5(a) Preparation of IgG from Anti-CR1 Antiserum and NRS

Before the preparation of $F(ab')_2$ fragments the IgG fraction had to be extracted from the serum. The principal of this method depends upon the observation that during anion exchange chromatography on DE52 run at pH7.6 in the absence of sodium chloride, IgG does not bind to the exchange resin but passes directly through the column. The only minor contaminants being P and C1q.

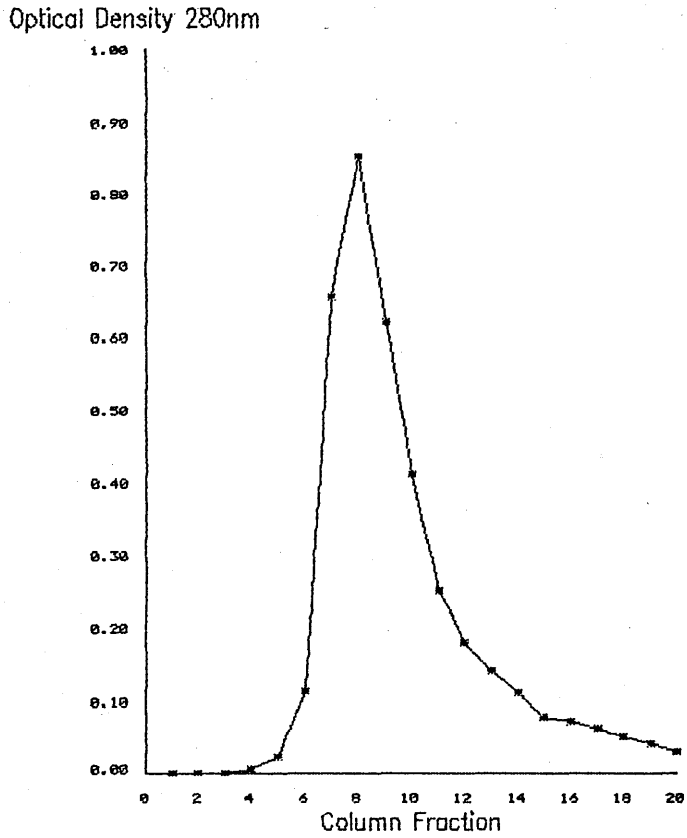
### Procedure

Ten millilitres of rabbit serum or anti-CR1 antiserum were adjusted to a pH of 7.6 by addition of hydrogen chloride prior to dialysis overnight at 4°C against 5 litres of phosphate buffer (0.01 mol $l^{-1}$ pH7.6). Following dialysis the pH and the conductivity of the serum were checked to ensure that the dialysis was complete. The dialysed antiserum was passed over a DE52 column and 5ml fractions were collected. The protein concentration of each fraction was then plotted (Fig 4.1) and the fractions containing a high IgG concentration were pooled.

Fig 4.1 DE52 Column

Legend

Purification of IgG fractions from rabbit serum by anion exchange chromatography using a 60ml DE52 column. The $OD_{280nm}$ of each 5ml fraction was plotted against the fraction number. The fractions containing the highest protein content (fractions 7-10) were pooled. The IgG content of these fractions was pure as determined by SDS-PAGE analysis.


Optical Density 280nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
2
4
6
8
10
12
14
16
18
20
Column Fraction

When the pooled fractions were analysed by double immunodiffusion, a technique described in chapter 2.6(f), against sheep anti-rabbit IgG a single precipitin line was observed which showed that the pool contained IgG. The purity of the IgG preparation was assessed by analysis on a 10% slab gel run under reducing and non-reducing conditions. Two bands were seen on reduced samples after silver staining (Plate 4.1). The molecular weights of these bands were 50,000 daltons and 25,000 daltons which are the molecular weights of the heavy and light chains of IgG respectively. A single band of 150,000 daltons was seen on the non-reduced sample (Plate 4.1). The protein concentration of the IgG pool was 0.7mg/ml. This was concentrated to 10mg/ml by pipetting the material into a dialysis sac and placing the sac into a large volume of sucrose (sucrose concentration).

4.5(b) Preparation of $F(ab')_2$ Fragments of IgG

The basis of this method is the digestion of IgG with pepsin at a low pH. Pepsin cleaves the IgG molecule between the $C_{H1}$ and $C_{H2}$ domains on the carboxy-terminal side of the interheavy-chain bridges to result in the production of a bivalent $F(ab')_2$ fragment and small peptide fragments derived from the remainder of the Fc fragments.

Procedure

Two millilitres of IgG (10mg/ml) from either NRS or anti-CR1 antiserum were dialysed against 5 litres of sodium acetate (0.1 mol $l^{-1}$ pH 4.5) overnight at 4°C. The pH of the IgG solution was adjusted to pH 4.5 (the optimal pH of pepsin) with acetic acid (0.1 mol $l^{-1}$). The IgG was warmed to 37°C and pepsin was added so that an enzyme to substrate ratio of 1:50(w/w) was achieved. The mixture was incubated overnight at 37°C after which insoluble material was removed by centrifugation (700g for 10 min at room temperature). The supernatant was collected with a Pasteur pipette and the pH adjusted to 7.4 using solid Tris base. Following dialysis against 5 litres of PBS the preparation was subjected to gel filtration chromatograhpy on Sephadex G-150.

4.5(c) Gel Filtration on Sephadex G-150

High molecular weight $F(ab')_2$ fragments (100,000 daltons) were separated from intact IgG and small degradation Fc peptides and pepsin on the basis of molecular weight on Sephadex G-150. Large molecules such as IgG (150,000 daltons) pass through in the void volume of the column while smaller peptides are retained in the gel and elute after the larger $F(ab')_2$ fragments.

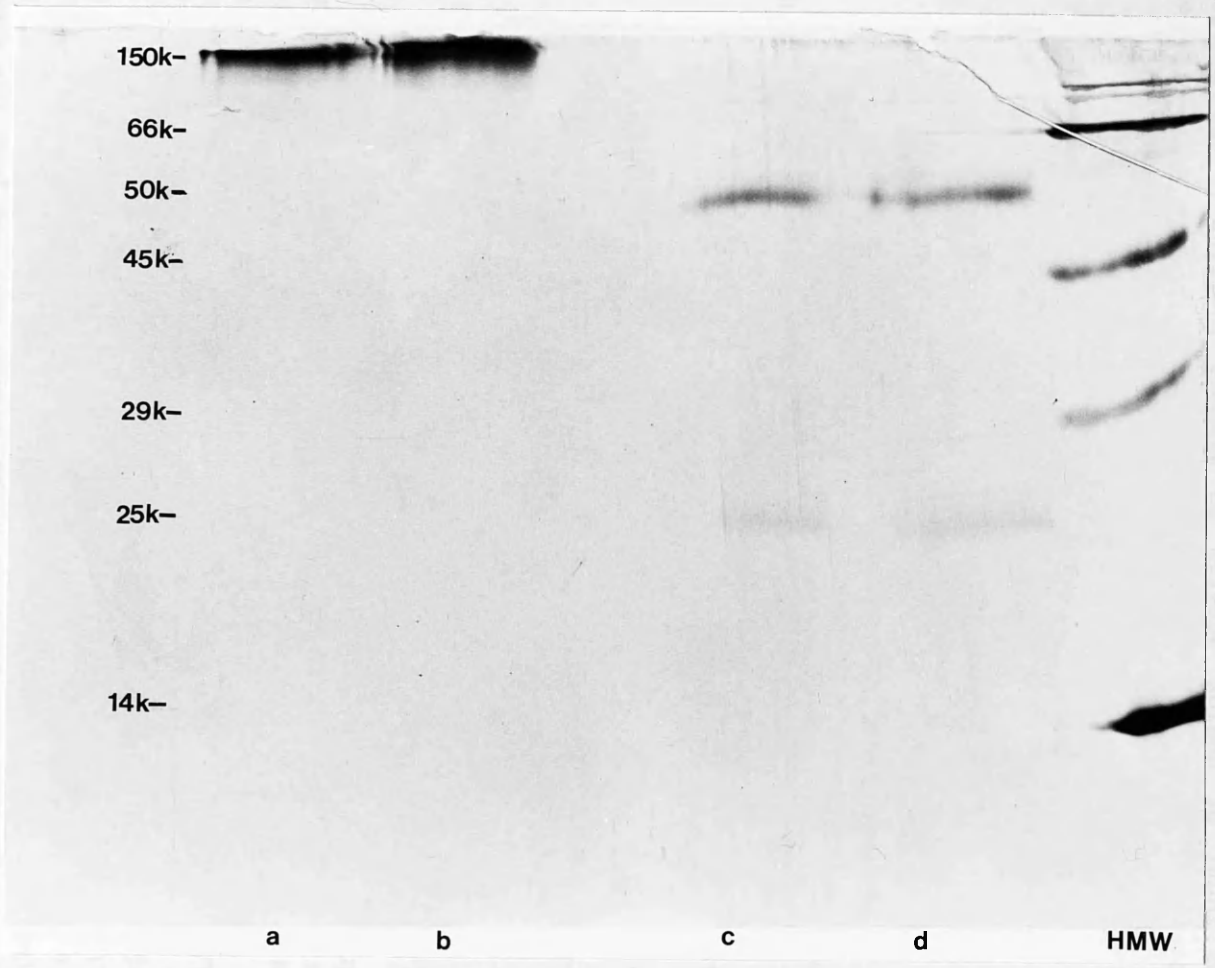
Procedure

The Sephadex G-150 column was washed with 1 column volume of PBS before the application of 3mls of pepsin digested IgG anti-CR1 or IgG NRS. One millilitre fractions were collected and following the measurement of the

Plate 4.1 SDS-PAGE Analysis of IgG

Legend

The purity of the IgG pool obtained from the DE52 column was assessed by analysis on a 10% slab gel. Samples were run under reducing and non-reducing conditions. In the reduced form 2 bands were seen after silver staining (tracks c and d). These bands had molecular weights of 25,000 and 50,000 daltons. They represent the light and heavy chains of the IgG molecule respectively. In the non-reduced form a single band with a molecular weight of 150,000 daltons was seen after silver staining (tracks a and b). This represents the whole IgG molecule.


150k–
66k–
50k–
45k–
29k–
25k–
14k–
a
b
c
d
HMW

protein content of each fraction the elution profile was plotted (Fig 4.2). This showed a major peak of protein which was $F(ab')_2$ and a subsidiary peak eluting later in the chromatogram. Fractions 47 to 53 were pooled. This pool had a protein concentration of 0.984mg/ml. When analysed by double immunodiffusion against sheep anti-rabbit $F(ab')_2$ a single precipitin line was observed which showed that the pool contained $F(ab')_2$ (Plate 4.2). A sample was also tested by immunoelectrophoresis and a single precipitin arc was formed between the purified $F(ab')_2$ and sheep anti-rabbit $F(ab')_2$ (1mg/ml). The $F(ab')_2$ fragments being more electronegative than whole IgG migrated further towards the anode than the larger IgG molecules (Plate 4.3). The purity of the $F(ab')_2$ preparation was determined by analysis on a 7½% tube gel run under non-reducing conditions. A single band was seen after staining with coomassie blue. The molecular weight of this band was 100,00 daltons which is the molecular weight of bivalent $F(ab')_2$ (Plate 4.4). This preparation contained no residual IgG.

4.6(a) Enumeration of E CR1 Numbers Using $F(ab')_2$ Anti-CR1

Reagents

Dibutylphthalate/dinoylphthalate

This solution was prepared as described in chapter 3.8(c).

$^{125}$I-Anti-CR1 $F(ab')_2$

$^{125}$I-anti-CR1 $F(ab')_2$ was prepared as described in chapter (2.6(d)) using the chloramine T method.

Buffers

RPMI/BSA

RPMI/BSA was prepared as described in chapter 3.6.

Procedure

Five millilitres of venous blood were transferred to a universal container containing 100µl of sodium heparin to prevent clotting. The E were separated from the plasma and buffy coat by centrifugation at 4,000g for 10 min at 4°C. The E were then washed 3 times in ice-cold RPMI/BSA and standardised to $2.5x10^6$/ml in RPMI/BSA. Triplicate aliquots (200µl) of E ($2.5x10^6$/ml) were transferred to a series of 8 microcap tubes. Unlabelled non-immune NRS $F(ab')_2$ fragments (12.5µg/tube) were added to tubes 1 to 4 and unlabelled anti-CR1 $F(ab')_2$ fragments (12.5µg/tube) were added to tubes 5 to 8.

The tubes were rotated end over end at 4°C for 1 hour on a Matburn mixer, after which incremental quantities of $^{125}$I-anti-CR1 $F(ab')_2$

Fig 4.2 Sephadex G-150 Column

Legend

Purification of $F(ab')_2$ fragments from anti-CR1 IgG by gel filtration using a 15x90cm Sephadex G-150 column. The $OD_{280nm}$ of each 1ml fraction was plotted against the fraction number. Two protein peaks were seen, the first of these contained $F(ab')_2$ fragments as determined by double immunodiffusion in agarose gels against sheep anti-rabbit $F(ab')_2$ and immunoelectrophoresis. Fractions 47 to 53 were pooled. The second peak contained smaller Fc degradation fragments.

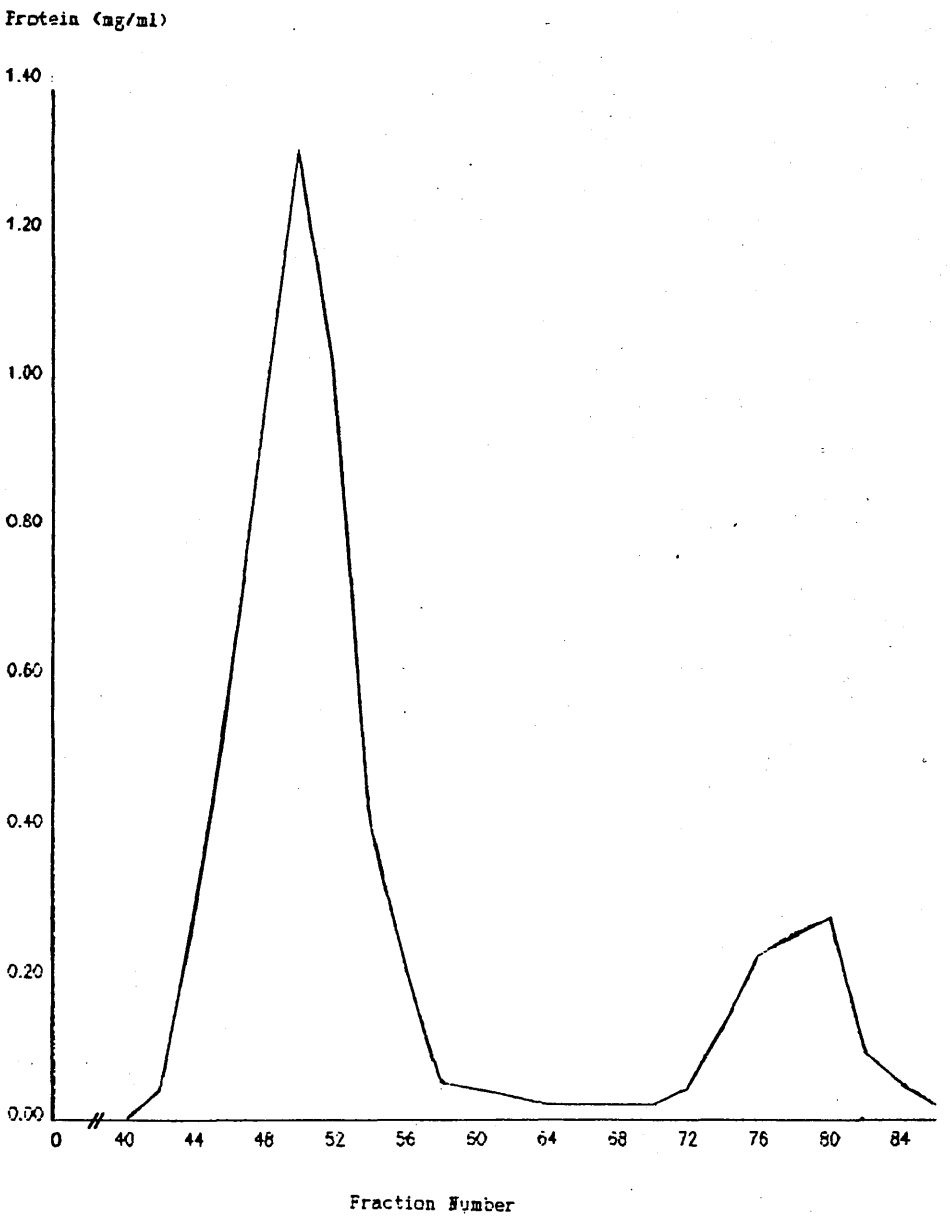

Protein (mg/ml)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
40
44
48
52
56
60
64
68
72
76
80
84
Fraction Number

Plate 4.2 Analysis of $F(ab')_2$ Fragments by Double Immunodiffusion in Agarose Gel

Legend

Double immunodiffusion in agarose gel of : A, $F(ab')_2$ fragments prepared from rabbit anti-CR1 IgG(1mg/ml) (wells 1 and 2) against sheep anti-rabbit $F(ab')_2$ (1mg/ml) (central well, a) and B, $F(ab')_2$ fragments prepared from normal rabbit IgG (1mg/ml) (wells 1 and 2) against sheep anti-rabbit $F(ab')_2$ (1mg/ml) (central well b). Single precipitin lines of identity were formed in all cases.

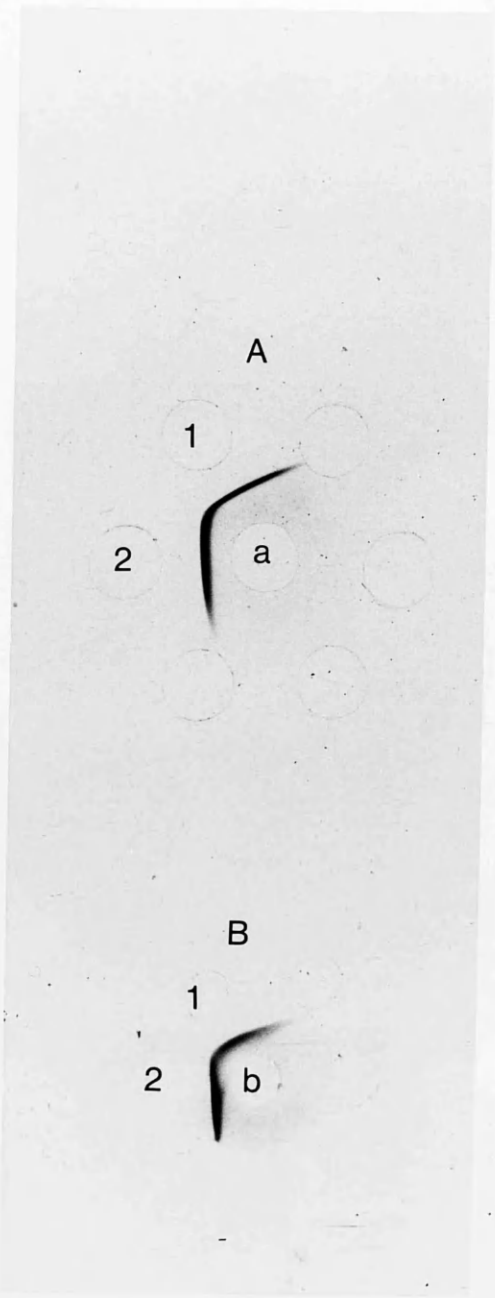

A
1
2
a
B
1
2
b

Plate 4.3 Analysis of IgG-Anti-CR1 and $F(ab')_2$ Anti-CR1 by Immunoelectrophoresis

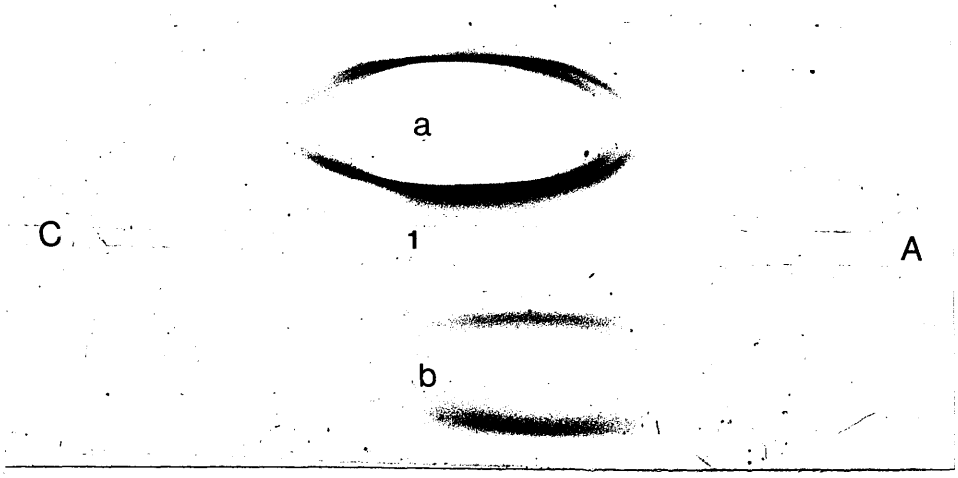
Legend

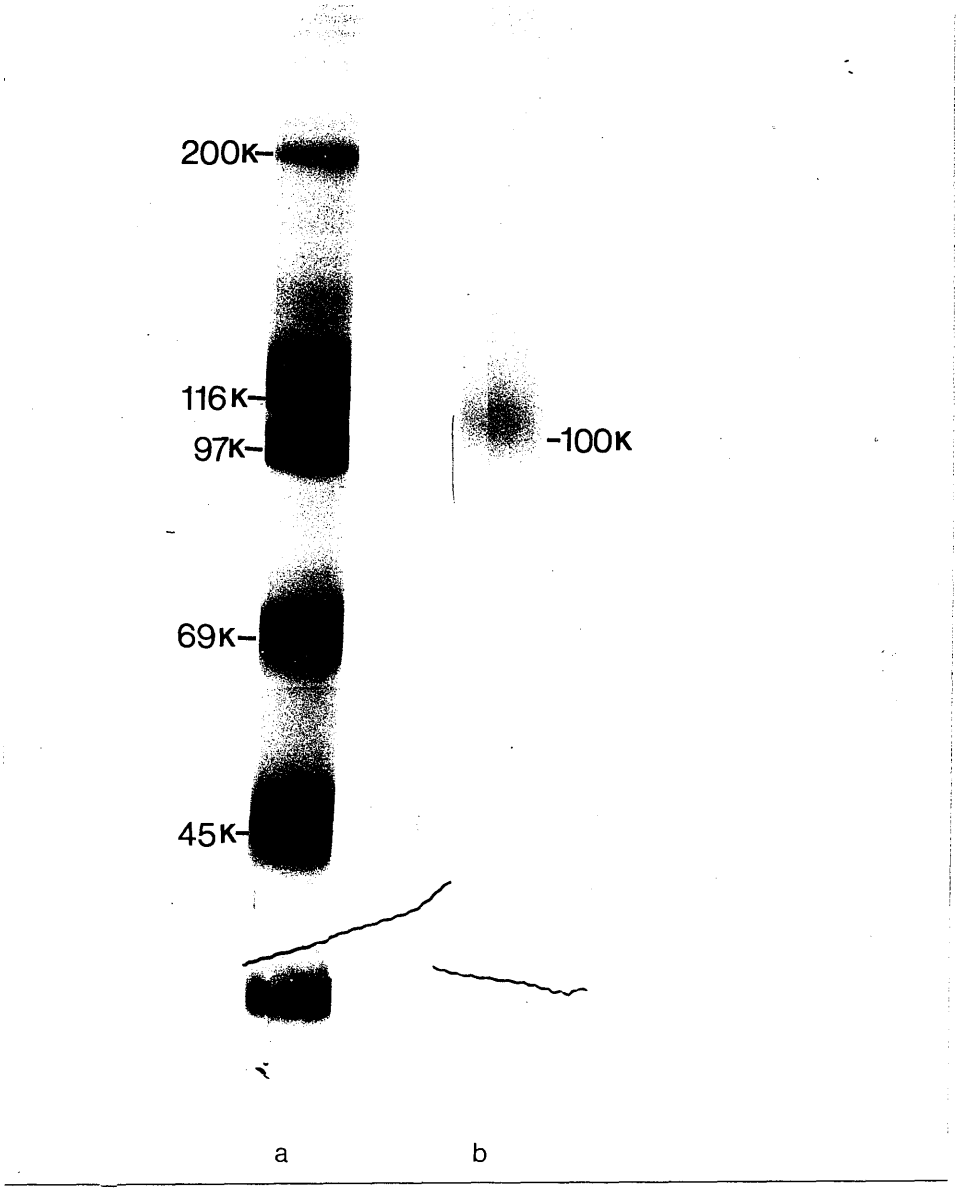
Immunoelectrophoretic analysis of whole IgG anti-CR1 (1mg/ml) (well a) and $F(ab')_2$ anti-CR1 (1mg/ml) (well b) against sheep anti-rabbit $F(ab')_2$ (1mg/ml) (trough 1). As the $F(ab')_2$ fragments were more electronegative than whole IgG, they migrated further towards the anode (A) than the whole IgG molecule which remained closer to the cathode (C).

Plate 4.4 SDS-PAGE Analysis of $F(ab')_2$ Anti-CR1

Legend

A sample of $F(ab')_2$ anti-CR1 from the pool obtained from the Sephadex G-150 column was analysed on a 7½% tube gel run under non-reducing conditions. After staining with coomassie blue a single band of molecular weight 100,000 daltons was revealed. This is the molecular weight of purified $F(ab')_2$ fragments. No contaminating proteins were observed.


a
C
1
A
b


200K
116K
97K
69K
45K
-100K
a
b

(1μg,2μg,3μg,4μg) were added to each set of 4 tubes. Following a further 1 hour incubation period at 4°C the samples were layered onto 300μl of a mixture of dibutylphthalate/dinoylphthalate in a microcap tube and the bound and free $^{125}$I-Fab'$_2$ separated by centrifugation at 10,000g for 2 min at room temperature. The supernatant was removed and the red blood cell pellet was cut from the bottom of the tube using a hot scalpel blade. The pellets were placed into glass carrier tubes and counted in an automatic γ counter.

4.6(b) Calculation of CR1 Number Using Scatchard Plot Analysis (Scatchard 1949)

The amount of radioactivity bound to E in the presence of non-immune F(ab')$_2$ fragments, which represents the total binding of $^{125}$I-anti-CR1 F(ab')$_2$ and the amount bound in the presence of excess unlabelled anti-CR1 F(ab')$_2$ fragments, which represents the nonspecific binding were plotted against the total input of $^{125}$I-anti-CR1 F(ab')$_2$ (Fig 4.3). Each point on the graph represents the mean ± the standard error of the mean (SEM) of 3 separate aliquots taken from the same sample and treated as 3 individual samples. The cpm obtained from all 3 samples were analysed and if they all fell within a 10% range of one another the mean ± SEM of all 3 was calculated. If however 1 sample was outwith this range it was discarded and the mean ± SEM of the remaining 2 was taken. If all 3 differed by more than 10% the mean ± SEM of all 3 was calculated.

The specific number of bound cpm were obtained by subtracting the lower nonspecific binding curve from the higher total binding curve and the number of free cpm were obtained by subtracting the specific number of bound cpm from the total radioactivity added to each tube. From this data a plot of bound cpm divided by free cpm against bound cpm was drawn (Fig 4.4). The point where this line crossed the Y axis was determined by linear regression analysis, with this point, (5450), representing the number of bound cpm to 200μl of the original E suspension in each tube, which comprises a total of 5x10$^7$E. The points on the Scatchard plot in Fig 4.4 were taken from the straightest part of the original total binding curve shown in Fig 4.3.

To convert the bound cpm obtained from the linear regression analysis into CR1 antigenic sites per cell the following calculations were performed.

The number of molecules in 1 cpm =

$$\frac{6.03\times10^{12}}{\text{number of cpm in 1}\mu\text{g of F(ab')}_2\text{ anti-CR1}}$$

The number of antigenic sites per cell =

$$\frac{\text{number of molecules in 1 cpm x number of bound cpm in 5x10}^7\text{ cells}}{5\times10^7}$$

Fig 4.3 Binding of $^{125}$I-Anti-CR1 F(ab')$_2$ To Erythrocytes

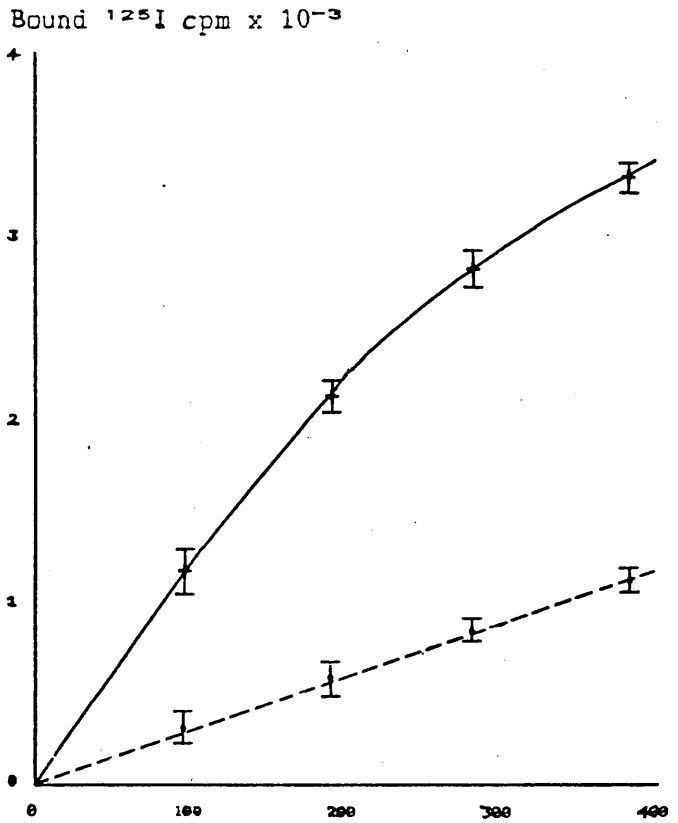
Legend

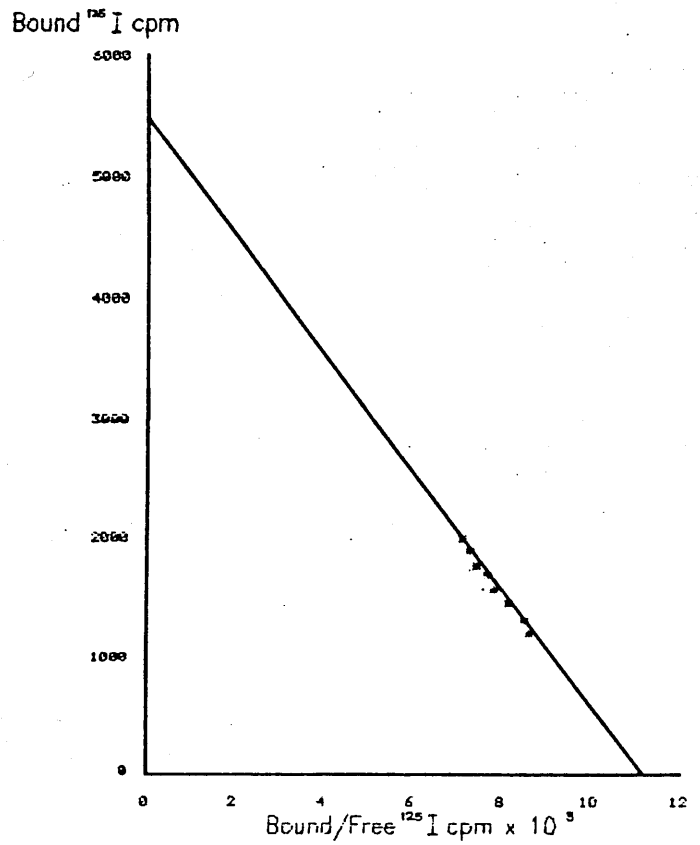
The amount of radioactivity bound to E in the presence of non-immune F(ab')$_2$ fragments (tubes 1-4) and the amount bound in the presence of anti-CR1 F(ab')$_2$ fragments (tubes 5-8) were plotted against the total input of anti-CR1 F(ab')$_2$ fragments, with each point being performed in triplicate (the vertical bars represent the SEM). The binding curves show the total amount of $^{125}$I-anti-CR1 F(ab')$_2$ bound (tubes 1-4) (x____x) and the nonspecific binding of $^{125}$I-anti-CR1 F(ab')$_2$ (tubes 5-8) (x----x). The ordinate axis shows the total input of $^{125}$I-anti-CR1 F(ab')$_2$ fragments (expressed as cpm) and the abscissa shows the total amount of $^{125}$I-anti-CR1 F(ab')$_2$ fragments which were bound (expressed as cpm).

Fig 4.4 Scatchard Plot

Legend

A Scatchard plot was constructed by plotting the number of specific bound $^{125}$I-anti-CR1 F(ab')$_2$ fragments (expressed as cpm) against the bound/free $^{125}$I-anti-CR1 F(ab')$_2$ fragments (expressed as cpm). The point where this line crosses the Y axis (5450) represents the number of bound cpm to $5x10^7$ E. The points taken for this graph were from the straightest part (from an input of 140,000 to 280,000 cpm of F(ab')$_2$ anti-CR1) of the total binding curve (Fig 4.3).


Bound $^{125}$I cpm x $10^{-3}$
4
3
2
1
0
0
100
200
300
400
Input $^{125}$I cpm x $10^{-3}$


Bound $^{125}$I cpm
5000
4000
3000
2000
1000
0
0
2
4
6
8
10
12
Bound/Free $^{125}$I cpm x $10^{3}$

The basis for this calculation was explained previously in chapter 3.8(d). In this case 1μg of $F(ab')_2$ molecules contains $6.03x10^{12}$ molecules. Having obtained the number of molecules in 1 cpm it was then possible to calculate the number of molecules contained in the number of bound cpm calculated from the linear regression analysis which was the total E input of $5x10^7$. Division of this number by $5x10^7$ resulted in the number of CR1 molecules (antigenic sites) per E.

## 4.7 Intra-Assay Variation

To assess the reproducibility of the assay a blood sample was taken from 1 donor and divided into 3 aliquots. Each aliquot was treated as a separate sample and the number of CR1 sites per E was calculated by Scatchard plot analysis. All of the assays were performed at the same time. Table 4.1 shows that the maximum intra-assay variation was ±5% of the mean.

## 4.8 Inter-Assay Variation

To assess the inter-assay variation a blood sample was collected from 1 donor and CR1 number determined immediately. The remainder of the blood which had not been used in the assay was standardised to $2.5x10^8$/ml in RPMI/BSA and stored overnight at 4°C. The following day the number of CR1 sites/E was determined. As is shown in Table 4.2 the maximum inter-assay variation was ±5% of the mean.

## 4.9 Stability of E CR1

To assess the stability of E CR1, E were standardised to $2.5x10^8$/ml in RPMI/BSA and CR1 sites/E calculated by RIA. The cells which were not used in the first assay were resuspended in either RPMI/BSA or a 1:4 dilution of autologous serum. These cells were kept overnight at 37°C. The following day they were centrifuged at 2,000g for 5 min at 4°C, the pellet was washed twice in ice-cold RPMI/BSA and the cells resuspended in RPMI/BSA at $2.5x10^8$/ml whereupon the number of CR1 sites were calculated by RIA.

## 4.10 Treatment of E with Proteolytic Enzymes

As discussed earlier removal of E CR1 by proteolytic enzymes is one of the methods whereby a reduction in CR1 numbers is thought to occur (4.1). To investigate this possibility E were treated with a number of enzymes.

### Procedure

Erythrocytes were standardised to $2.5x10^8$/ml in RPMI/BSA. The concentrations of enzymes shown in Table 4.3 were added to 5mls of the E suspension and the cells incubated for 1 hour at 37°C in a shaking water bath. Following this incubation the cells were centrifuged at 2000g for 5 min at 4°C, the supernatant was removed and the cell pellet washed twice

Table 4.1 Intra-Assay Variation

CR1 Sites/E

| | First Measurement | Second Measurement | Third Measurement | Mean±SD | % Variation from Mean |
|---|---|---|---|---|---|
| Donor 1 | 1255 | 1248 | 1298 | 1267±27 | +2 -2 |
| Donor 2 | 4341 | 4610 | 4474 | 4475±134 | +3 -3 |
| Donor 3 | 5092 | 5600 | 5353 | 5348±254 | +5 -5 |

Note

The mean±SD was calculated for each set of measurements performed on the 1 donor. The highest and lowest CR1 numbers in each set of measurements were then expressed as a percentage of the mean. This result is tabulated in the end column (% variation from the mean).

Table 4.2 Inter-Assay Variation

CR1 Sites/E

| | First Measurement | Second Measurement | Mean±SD | % Variation from Mean |
|---|---|---|---|---|
| Donor 1 | 3952 | 3548 | 3753±289 | +5 -5 |
| Donor 2 | 1434 | 1563 | 1498±91 | +4 -4 |
| Donor 3 | 1785 | 1983 | 1884±140 | +5 -5 |

Note

The first measurement was performed on freshly isolated E, while the second measurement was performed after overnight incubation of the E at 4°C in RPMI/BSA. The mean±SD for the 2 measurements was calculated and then the percentage variation from this mean for each of the 2 numbers was determined.

Table 4.3 Treatment of E with Proteolytic Enzymes

| Enzyme | Concentration |
|---|---|
| Plasmin | 9.1μg/ml |
| Thrombin | 5 units/ml |
| Kallikrein | 1.8μg/ml |
| Cathepsin C* | 9.09μg/ml |
| Cathepsin D* | 9.09μg/ml |
| Elastase | 54.4μg/ml or 2.27mg/ml |
| Trypsin | 1mg/ml, 0.01mg/ml or 0.0001mg/ml |

Note

* The RPMI/BSA was made to a pH of 5 (the optimal pH for these 2 enzymes) using hydrochloric acid (0.1 mol $l^{-1}$).

in ice-cold RPMI/BSA and the cells restandardised to $2.5x10^8$/ml. In the experiments where trypsin was used a 2-fold molar excess of soybean trypsin inhibitor was added at the end of the 1 hour incubation. The cells were incubated for a further 2 min and then treated as described above.

Determination of Enzyme Concentration

As none of the proteolytic enzymes used in the study are normally present in serum under physiological conditions a concentration within the physiological range of their proenzyme was selected. As there are approximately $5.5x10^9$ E in 1ml of packed red cells the proenzyme concentration was divided by 4.4 to adjust the concentration for the number of cells used in the assay ($1.25x10^9$/5mls).

## 4.11 Effect of SLE Sera on E CR1

This study was undertaken to investigate the possibility that some component present in the sera of SLE patients could remove CR1 from E or block CR1. Blood was taken from donors who had the blood group O and who were rhesus positive ($O^{+ve}$) to prevent lysis of the E due to complement activation upon addition of heterologous serum.

Procedure

Ten millilitres of blood were collected and 8mls of this was allowed to clot at 37°C for the preparation of autologous serum as was described previously in chapter 2.6(a). The remaining 2mls were mixed with sodium heparin and the E isolated and standardised to $2.5x10^8$/ml in RPMI/BSA. Erythrocyte CR1 numbers were then calculated by RIA. The remaining blood not used in the assay was re-standardised to $1x10^9$/ml in SLE serum (diluted 1:4 in RPMI/BSA) from patients who had active or inactive disease at the time of venous blood sampling. Cells were also resuspended in pooled heterologous normal serum (diluted 1:4 in RPMI/BSA).

This number of cells and this dilution of serum were used as they approximate to a physiological ratio of E to plasma. The blood was then stored overnight at 37°C in an incubator. The following morning the E were centrifuged at 2000g for 5 min at 4°C the supernatant was removed and the E were then washed 3 times in ice-cold RPMI/BSA, standardised to $2.5x10^8$/ml and the number of CR1 sites per E calculated by RIA.

## 4.12 Preparation of C3b Coated IgG Aggregates

One of the possible mechanisms for the reduction of E CR1 in SLE patients is the blockade of the receptor by large opsonised complexes.

In order to study this possibility it was decided to prepare such complexes and incubate them with E and then measure residual CR1 sites.

Reagents

Human plasma was supplied by the Blood Transfusion Service Law Hospital Carluke Scotland.

Buffers

Saturated ammonium sulphate was prepared as described in Chapter 3.8(a).

Phosphate Buffer

Phosphate buffer (0.01 mol $l^{-1}$ pH 7.6) was prepared as described in Chapter 2.8

5xVBS

5xVBS was prepared as described in Chapter 2.7(b).

4.12(a) Lyophilisation

The sample was put into a round bottomed flask and quickly frozen by placing the flask into a mixture of methanol and dry ice. The flask was attached to a freeze drier and dried under vacuum for 5 hours. The protein precipitate was redissolved in deionised water.

4.12(b) Sucrose Gradients

Sucrose solutions, 10%(w/v), 20%(w/v), 30%(w/v) and 40%(w/v) were made up in deionised water after which BSA (0.5%(w/v)) was layered on top and left to dissolve. The sucrose solutions were chilled on ice to 0°C. Using a 1ml syringe the 10% solution was added to an ultracentrifuge tube which was also on ice. The 20% solution was then carefully underlayed by gently injecting 1ml of the solution under the 10% solution. Following this the 30% and finally the 40% solutions were underlayed in a similar manner. All solutions were kept on ice.

4.12(c) Preparation of IgG from Human Plasma

One pack of expired plasma (250mls) was thawed at 37°C and placed into a volumetric flask. The plasma was allowed to clot by the addition of 4mls of calcium chloride (1 mol $l^{-1}$) and the mixture stirred overnight at 4°C. The serum was separated from the clot by filtration through a Buchner funnel. An equal volume of SAS was added dropwise to the serum which was being constantly stirred at room temperature. Stirring was continued for 30 min following the addition of the SAS. The precipitate was removed by centrifugation at 1000g for 25 min at 4°C and redissolved in 5xVBS. This was then dialysed against 24 litres of phosphate buffer (0.01 mol $l^{-1}$ pH 7.6) for 2 days at 4°C and then passed over a DE52 column which had been equilibrated with the same buffer. The protein concentration of each fraction was measured spectrophotometrically at an absorbance of

280nm and the exclusion peak was pooled and tested for IgG concentration and purity as described earlier (4.5(c)). Sheep anti-human IgG was used in the double immunodiffusion analysis instead of sheep anti-rabbit IgG.

4.12(d) Preparation of Alkali Aggregated IgG

Purified human IgG was lyophilised and reconstituted in deionised water to give a final concentration of 20mg/ml. To 5mls of the IgG solution, 5mls of sodium hydroxide (0.2 mol $l^{-1}$) were added and the mixture was immediately dialysed against 5 litres of PBS pH7.2 at room temperature for 2 hours. The buffer was then changed and the dialysis continued overnight at room temperature.

The aggregates (200µl) were applied to 10% to 40%(w/v) sucrose gradients (4ml) containing 0.5% BSA(w/v) and subsequently ultracentrifuged for 16 hours at 288,000g in a SW41 Beckman rotor. After centrifugation the gradients were fractionated into 15 fractions using a Beckman fraction recovery system which punctures the bottom of the ultracentrifuge tube. Thus the fractions containing the larger aggregates are collected before the smaller aggregates which do not sediment as quickly. These fractions were dialysed against PBS overnight at 4°C to remove the sucrose and tested for protein concentration by Folin analysis as described in chapter 2.9 (Fig 4.5). The first 6 fractions containing the largest aggregates and the highest protein concentration (1.5mg/ml) were pooled.

4.12(e) Opsonisation of Aggregated IgG with C3b

The pool of aggregates were divided equally into 2 aliquots. To 1 of these aliquots an equal volume of fresh NHS was added and the mixture was incubated for 20 min at 37°C. These aggregates were separated from free C3 and C3 degradation products by ultracentrifugation at 288,000g for 1 hour at 4°C on 10% to 40% discontinuous sucrose gradients containing 0.5%(w/v) BSA. The gradients were collected in 20 fractions which were then dialysed against PBS to remove the sucrose. Their protein concentrations were tested by Folin analysis as described in chapter 2.6(c). Each fraction was then tested for C3b content by its ability to agglutinate human E.

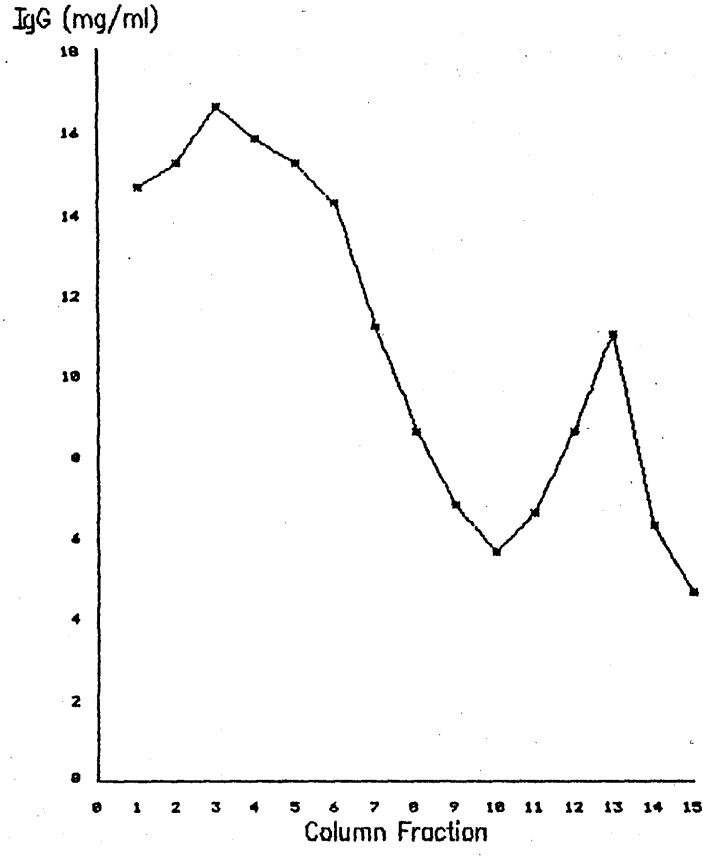
4.12(f) Agglutination of Human E

Human E were washed 3 times in RPMI/BSA and standardised to $2.5x10^{8}$/ml. Each fraction from the sucrose gradient was then serially diluted to a 1:256 dilution in a microtitre plate. Erythrocytes (50µl) were added and the was plate left to settle for 3 hours after which the agglutination pattern was noted. Controls using unopsonised IgG aggregates showed no agglutination while coated aggregates caused haemagglutination at up to a 1:8 dilution. The agglutination of E by C3b coated aggregates could be inhibited by addition of 50µl of the C3b coated aggregates. This proved that the agglutination produced by the serum-treated aggregates was due to the binding of aggregate-bound C3b to E CR1.

Fig 4.5 Protein Content of Alkali Aggregated IgG

Legend

The protein content of the aggregated IgG fractions from the sucrose gradients were determined by Folin analysis and plotted against their fraction number. The first 6 fractions containing the heaviest IgG aggregates and the highest protein content were pooled. Fractions 10-15 contained monomeric IgG.


IgG (mg/ml)
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Column Fraction

4.12(g) Measurement of CR1 Antigenic Sites in the Presence of Aggregates

Human E (4.8mls) $2.5x10^8$/ml were incubated with C3b coated aggregates (at the predetermined highest dilution which caused agglutination) at 37°C for 1 hour with end over end mixing. The same volume of E were incubated with the same dilution of uncoated IgG aggregates from the pool described in 4.12(d). After 1 hour 200µl aliquots of E from both sets of cells were transferred to microcap tubes and the number of CR1 antigenic sites was measured as before (4.6(a)).

4.13(a) Modulation of E CR1 Expression in Response to Oral Antigen (Cows Milk) Challenge

High levels of circulating immune complexes have been detected following food consumption in some normal individuals (Paganelli et al 1979). Sandilands et al (1982) found that after consumption of 1.2 litres of cows milk by normal human adults that there was a rapid fall in the Fc γ receptors on peripheral blood lymphocytes. It was thus decided to investigate the possibility that food antigens could block CR1 expression on E.

Procedure

Following an overnight fast 5 normal males and 1 normal female drank 1.2 litres of cows milk over a period of 10 min. Heparinised (2mls) and clotted (5mls) blood samples were collected immediately before milk consumption and at 5 intervals thereafter. The heparinised samples were washed and standardised to $2.5x10^8$/ml in RPMI/BSA and incubated on ice until all the samples had been collected. Thereafter CR1 number was determined on each aliquot using the monoclonal antibody E11 as described earlier in chapter 3.8(c). The serum for each time interval was stored at -70°C until required. The control for this experiment involved an overnight fast with no milk consumption. Heparinised and clotted blood samples were then collected at similar time intervals to those in the previous experiment.

4.13(b) Incubation of E with Sera Collected from Donors after Ingestion of 1.2 Litres of Cows Milk

To investigate the possibility that immune complexes had formed after milk consumption, E from a donor with the blood group O who was rhesus positive ($O^{+ve}$)were incubated with the serum from each of the different time intervals before and after milk consumption.

Procedure

Erythrocytes from an $O^{+ve}$ donor were standardised to $2.5x10^8$/ml in RPMI/BSA and aliquots (200µl) were placed into microcap tubes. These tubes were centrifuged for 30 seconds at 10,000g at room temperature and the supernatant removed. Neat sera (9µl) from each time point in the milk

experiment were added to a set of 6 E pellets. The E were resuspended in the serum and incubated for 30 min at 37°C after which 200µl of RPMI/BSA were added to each microcap. To 3 of the sets of E for each time point 700ng of $^{125}$I-E11 were added and to the remaining 3 sets a 10-fold molar excess of non-radioactive E11 was added. The number of CR1 sites per E was then determined as previously described in chapter 3.8(c).

4.14(a) Radioiodination and Immunoprecipitation of E CR1

As an alternative means of studying E CR1 it was decided to label E CR1 with $^{125}$I. This provided a means of visualising CR1 on autoradiographs and gave an indication of any change in CR1 structure which may be brought about by treatment with serum enzymes or patient's serum.

Addition of D-glucose to E in the presence of $^{125}$I, lactoperoxidase and glucose oxidase, results in the formation of small amounts of hydrogen peroxidase ($H_2O_2$) at a steady rate. The lactoperoxidase in turn catalyses the oxidation of the radiolabelled iodide to iodine which reacts with the E surface protein. This reaction may be summarised by the following equation.

$$\beta\text{-D Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} = H_2O_2 + \text{Gluconalactone}$$

$$H_2O_2 + {}^{125}I^- + \text{protein} \xrightarrow{\text{lactoperoxidase}} = \text{Iodoprotein}$$

Reagents

Lactoperoxidase

Lactoperoxidase was stored desiccated at -20°C. Prior to use a 1mg/ml solution was made up in deionised water.

Glucose Oxidase

Glucose oxidase was stored at 4°C in acetic acid buffer at 12000 units/ml

D-Glucose

A 50mg/ml D-glucose solution was made up in deionised water on the day before use to allow rotation to the β form.

Protein A Sepharose

A protein A crude cell suspension (10%w/v) of Staphylococcus aureus (Cowan strain) cells was stored in potassium phosphate buffer (0.05 mol $l^{-1}$ pH 7.5) at 4°C. Prior to use the suspension was centrifuged at 10,000g for 2 min at room temperature and the pellet resuspended in 1ml of PBS. The pellet was washed twice in PBS and finally resuspended to a 10%(w/v) suspension in PBS.

Destain Solution

Destain solution was prepared as previously described in chapter 2.6(f).

Buffers

Water/Protease Inhibitors

Deionised water containing EDTA (3 mmol $l^{-1}$) iodoacetimide (0.02 mol $l^{-1}$) PMSF (2 mmol $l^{-1}$) and 0.33 units/ml aprotonin

Sodium Chloride/Protease Inhibitors

Sodium chloride (0.3 mol $l^{-1}$) containing the same proteases as above.

1% NP-40/PBS

1ml of NP-40 was mixed with 100mls of PBS.

1% NP-40/PBS/Protease Inhibitors

1% NP-40/PBS containing EDTA (3 mmol $l^{-1}$), IA (0.02 mol $l^{-1}$), PMSF (2 mmol $l^{-1}$), 0.33 units/ml aprotonin, pepstatin A (25 $\mu$mol $l^{-1}$).

Dissociating Buffer

3mls of 10%(w/v) SDS, 0.85mls of Tris (0.75 mol $l^{-1}$ pH6.8), 1ml of glycerol, 150$\mu$l of bromophenol blue and 5mls of deionised water were mixed together.

Sample Buffer
Dissociating buffer was made up containing 0.5mls of 2-mercaptoethanol.

Procedure

Ten millilitres of venous blood were mixed with 160 units of sodium heparin in a universal container and centrifuged at 2000g for 8 min at 4°C and the plasma and buffy coat removed by aspiration. The cells were then washed 4 times in PBS and standardised to $1.25 \times 10^{9}$/ ml in PBS and 4mls were transferred into a glass bijoux bottle containing 120$\mu$l of lactoperoxidase, 25$\mu$l of potassium iodide (0.3 mmol $l^{-1}$) and 5$\mu$l of glucose oxidase, $^{125}I$ (0.25mCi) and 120$\mu$l of $\beta$-D glucose were then added. The reaction mixture was rotated end over end on a Matburn mixer for 15 min at room temperature after which the reaction was stopped by the addition of L-tyrosine to a final concentration of 2 $\mu$mol $l^{-1}$. The E were washed 3 times in ice-cold PBS by centrifugation at 2000g for 5 min at 2°C. The E were chilled on ice for 15 min and then lysed by the addition of 15mls of ice-cold water/protease inhibitors. Two minutes later 15mls of sodium chloride/protease inhibitors were added to restore isotonicity. The lysate was centrifuged at 33,000g for 30 min at 4°C and following the

removal of the supernatant the membrane pellet was resuspended in 1ml of 1% NP-40/PBS. The solution was incubated on ice for 60 min to allow solubilisation of CR1. Erythrocyte CR1 was collected by centrifugation of the solubilised membranes at 10,000g for 30 min at 4°C. The clear supernatant containing CR1 was removed and stored at -70°C and the membrane pellet discarded.

4.14(b) Immunoprecipitation of Radiolabelled CR1 from Solubilised E Membranes

The basis of the procedure is to add anti-CR1 to the $^{125}I$ labelled solubilised membranes. CR1 binds to the antibody and can be removed from the mixture by binding to a protein A cell suspension. In preliminary experiments it was found that the monoclonal anti-CR1 did not bind to the protein A, therefore a second antibody, the IgG fraction of rabbit anti-mouse globulin, was added to the incubation mixture before the addition of the protein A cell suspension.

Procedure

To reduce the problem of nonspecific binding of membrane proteins to protein A, the iodinated lysates were preabsorbed with 25µl of a protein A cell suspension by incubation for 30 min at 4°C with end over end mixing. The pellet was collected by centrifugation at 10,000g for 2 min at room temperature and the supernatant was removed and divided equally between 2 microcap tubes. To 1 of these tubes 5µl of normal mouse serum was added . This was designated the control tube. To the second microcap 5µl of monoclonal anti-CR1 IgG was added. This was designated the specific tube. These 2 tubes were incubated for 1 hour at 4°C with mixing, followed by addition of 5µl of a second antibody, rabbit anti-mouse IgG (2.4mg/ml). The tubes were incubated for a further 1 hour at 4°C after which 25µl of a protein A cell suspension were added and the incubation continued for 30 min at 4°C. The pellet was collected by centrifugation and washed 5 times with a 1% NP-40/PBS buffer. An aliquot of dissociating buffer (40µl) was then added to each pellet which was mixed thoroughly and set in boiling water for 2 min . The pellets were centrifuged at 10,000g for 2 min at room temperature and the supernatants were removed and diluted with an equal volume of sample buffer containing 2-mercaptoethanol. These were then subjected to SDS-PAGE on 5% slab gels. When the electrophoresis was complete the gel was incubated for 30 min in destain solution to fix the protein. The gel was then transferred to a slab gel drier and dried under vacuum for 2 hours at 70°C. After drying, the gel was removed and placed in an X-ray cassette. A sheet of X-ray film was placed on top of the gel and the cassette stored in the dark at room temperature for the required time. After this the film was removed and developed using Kodak developer and fixer according to the manufacturers instructions.

## 4.15 Separation of CR1 from Solubilised E Membranes by Affinity Chromatography

It was decided to use an alternative method of isolating CR1 from E for 2 reasons, (i) to see if the protein immunoprecipitated by the monoclonal anti-CR1 antibody was the same as the protein isolated by affinity chromatography on C3-Sepharose and (ii) to determine if this method would be quicker or more convenient than the alternative means.

### Reagents

#### IgG Sepharose

IgG Sepharose was prepared by Dr W Mitchell.

#### C3-Sepharose

C3-Sepharose was prepared as described in chapter 2.9(b).

#### Borate Buffer

Borate buffer was prepared as described in chapter 2.9(b).

#### Elution Buffer

Acetic acid (0.1 mol $l^{-1}$), sodium chloride (0.4 mol $l^{-1}$) and 1ml of NP-40 were mixed together in 100mls of deionised water.

#### Neutralisation Buffer

Tris HCl (2 mol $l^{-1}$ pH 8.6) and 1ml of NP-40 were made to a final volume of 100mls in deionised water.

### Procedure

Solubilised membranes from $2x10^{10}$ E were incubated with end over end mixing on a Matburn mixer at room temperature for 30 min with 400µl of IgG-Sepharose to absorb nonspecific proteins. The solution was centrifuged at 300g for 5 min at room temperature and the supernatant removed and diluted with 2 parts of deionised water containing 1% NP-40 to reduce the conductivity of the solution. Half of this solution was added to 400µl of C3-Sepharose and half was added to 400µl of IgG-Sepharose to provide a control for nonspecific binding. After a 1 hour incubation with end over end mixing on a Matburn mixer at room temperature the samples were centrifuged at 300g for 5 min at room temperature and the supernatants removed. Six millilitres of borate buffer were added to the beads which were then transferred to a 2ml syringe barrel and washed with 4mls of the same buffer. Each of the 1ml acid eluates were collected into microcap tubes containing 125µl of neutralising buffer for immediate neutralisation. Each eluate was

precipitated with 5 volumes of acetone and after a 5 min incubation at room temperature the samples were centrifuged for 5 min at 10,000g at room temperature. The supernatants were discarded and the pellets redissolved in Tris HCl (2 mol $l^{-1}$ pH 6.8). The protein was reprecipitated with acetone by mixing with 5 volumes of acetone for 5 min at room temperature followed by centrifugation at 10,000g for 5 min at room temperature. The supernatant was removed and the pellet dissolved in 40µl of dissociating buffer prior to SDS-PAGE analysis on 5% slab gels.

4.16 Stability of E CR1

The stability of E CR1 was assessed after the E had been surface labelled by incubating the labelled E for a particular time interval before lysing the cells and immunoprecipitating CR1. If the receptor was shed within a certain time interval then the radiolabelled CR1 would be lost and would not show up on autoradiographs.

4.17 Trypsinisation of Surface Labelled E

After having surface labelled E with $^{125}I$ it was possible to study how the expression of CR1 was affected by treatment with various concentrations of trypsin. This particular study had 2 aims, (i) to discover if the concentration which removed all CR1 detectable by RIA was in fact removing all CR1 and (ii) to investigate the possibility that regeneration of E CR1 could occur after trypsinisation.

Procedure

Erythrocytes were standardised to $1.25x10^9$/ml in PBS and warmed to 37°C. Four millilitres of the appropriate concentration of prewarmed trypsin solution (0.1mg/ml, 0.01mg/ml, 0.005mg/ml, 0.001mg/ml or 0.0001mg/ml) were added to 4mls of E in a universal container. The cells were incubated at 37°C for 20 min after which a 2-fold molar excess of SBTI was added. Incubation was continued for 2 min, the cells were then centrifuged at 2,000g for 5 min at 4°C. The cell pellet was washed 3 times in ice-cold PBS. The cells were finally resuspended in 4mls of PBS, surface labelled and CR1 immunoprecipitated.

In order to assess if trypsinised E could regenerate CR1, after the E were trypsinised, 1 aliquot was surface labelled and immunoprecipitated immediately. The remaining aliquots were incubated in either PBS, RPMI/BSA or plasma for different time intervals at 37°C and then radiolabelled, lysed and immunoprecipitated for CR1.

4.18 Treatment of Radiolabelled E with SLE Sera and RA Sera

After surface labelling the E the cells were not lysed but were incubated overnight at 37°C in 1:4 dilutions of normal sera or sera from either SLE patients or RA patients. Following this incubation the E were lysed as before, the membranes solubilised and CR1 immunoprecipitated.

Results

4.19 Determination of E CR1 Number

Erythrocyte CR1 could be measured successfully by polyclonal anti-CR1 antiserum. The maximum intra-assay variation was ±5% of the mean (Table 4.1) which indicated that the assay was reproducible. The range of E CR1 numbers obtained from 27 individuals was large (1,255 to 33,854) with a mean value of 3,773. A large range of E CR1 levels has also been found by others using polyclonal anti-CR1 to quantify CR1 (Wilson et al 1982; Holme et al 1986).

The maximum inter-assay variation was ±5% of the mean (Table 4.2). The fact that this variation in CR1 number was small after storage overnight at 4°C in RPMI/BSA allowed E suspensions to be stored on ice for a number of hours or overnight at 4°C before measuring CR1 numbers.

4.20 Stability of E CR1

The results from the inter-assay variation gave some indication that E CR1 was stable and was not affected by overnight storage at 4°C. To determine if E CR1 numbers were stable at 37°C, E were incubated overnight at 37°C in RPMI/BSA or autologous serum (Table 4.4). This storage overnight at 37°C did not affect E CR1 number.

4.21 Effect of Proteolytic Enzymes on E CR1 Levels

Erythrocyte CR1 levels were calculated on E after incubation with proteolytic enzymes at the concentrations indicated in Table 4.5. As can be seen from this table, when the physiological concentrations of these enzymes were used none of the enzymes reduced CR1 levels. However, high concentrations of elastase (2.27mg/ml) and trypsin (1 and 0.01mg/ml) removed all CR1 detectable by anti-CR1 and at a trypsin concentration of 0.1µg/ml, E CR1 receptors were reduced by 40%.

4.22 Effect of SLE Sera on E CR1 Levels

It is possible that some proteolytic component in SLE sera is able to remove or block CR1. Experiments were therefore performed in which E from $O^{+ve}$ blood group donors were incubated overnight at 37°C in sera from patients with active or inactive SLE. The results of this study are shown in Table 4.6. As can be seen, CR1 numbers fell most after an overnight incubation in pooled heterologous sera. Erythrocyte CR1 numbers fell to a lesser extent in sera from a patient with active SLE.

Table 4.4 Stability of E CR1 after Storage Overnight at 37°C

| Medium for Overnight Incubation | Number of Samples | Mean percentage Change from Control±SE |
|---|---|---|
| RPMI | 3 | -4±22 |
| 25% Autologous Serum | 3 | -9±14 |

Note

CR1 levels were measured on the E of 3 individuals immediately after isolation or after storage of the E in RPMI or 25% autologous serum overnight at 37°C. The change in E CR1 number after storage was calculated as a percentage of the E CR1 number on freshly isolated E (control). The mean percentage change of the 3 individual donors was then calculated.

Table 4.5 Effect of Proteolytic Enzymes on E CR1

| Enzyme | Concentration | Mean percentage Change from Control |
|---|---|---|
| Thrombin | 5 units/ml | 26±18 |
| Elastase | 45μg/ml | -14±2 |
| Elastase | 2.27mg/ml | -100±0 |
| Kallikrein | 1.8μg/ml | -5±9 |
| Cathepsin C | 9.09μg/ml | +1±16 |
| Cathepsin D | 9.09μg/ml | -4±15 |
| Plasmin | 9.1μg/ml | -11±1 |
| Trypsin | 1mg/ml | -100±0 |
| Trypsin | 0.01mg/ml | -100±0 |
| Trypsin | 0.0001mg/ml | -40±21 |

Note

E CR1 levels were measured on E after incubation with the enzymes tabulated above. The change in E CR1 number caused by each enzyme was then expressed as a percentage of a control E sample from the same donor which had not been exposed to the enzyme. Each enzyme was incubated with E from 3 different donors and the mean percentage change from the control was determined.

Table 4.6 Effect of SLE Sera on E CR1

| Medium for Overnight Incubation | Number of Samples | Mean Percentage Change from Control ±SE |
|---|---|---|
| 25% Heterologous Normal Serum | 2 | -40±20 |
| 25% Active SLE Serum | 4 | -27±18 |
| 25% Inactive SLE Serum | 5 | +5.2±24 |

Note

CR1 levels were measured on freshly isolated E (control) and on E which had been incubated overnight in the 3 types of sera indicated above. The change in E CR1 number caused by the incubation was expressed as a percentage of the control. The mean percentage change from the control was then determined for each group of samples.

4.23 Measurement of CR1 Numbers in the Presence of Complement Coated IgG Aggregates

The number of CR1 detectable on the E of 2 donors fell considerably in the presence of large opsonised IgG aggregates as compared with the measurement in the presence of large unopsonised IgG aggregates (Table 4.7).

4.24 Modulation of E CR1 Expression in Response to Oral Antigen Challenge

Erythrocyte CR1 levels were measured before and after oral antigen challenge. The results are tabulated in Table 4.8 and presented graphically in Fig 4.6. The maximum drop in E CR1 levels was achieved 2 hours after drinking 1.2 litres of cows milk. By 4 hours the E CR1 levels had returned to normal. Two control experiments were performed in which blood samples were collected after an overnight fast. These individuals did not drink any milk and no food was consumed until the last blood sample had been removed. As can be seen from Table 4.9, CR1 levels remained constant at all time points.

In order to determine if there were antigen/antibody complexes in the serum which could block E CR1 expression the sera from individuals who had ingested 1.2 litres of cows milk were incubated with E from $0^{+ve}$ blood group donors. Erythrocyte CR1 number was then determined. The results showed that the sera do not produce E CR1 blockade (Table 4.10).

4.25 Immunoprecipitation of CR1 from E

Erythrocytes were radiolabelled and the membranes collected and solubilised. CR1 was immunoprecipitated from the supernatant of these solubilised membranes using both a commercially available monoclonal anti-CR1 antibody and the IgG fraction of the antiserum to purified CR1. Both of these antisera precipitated a band of molecular weight 220,000 daltons (Plate 4.5). A band of similar molecular weight was separated from solubilised membranes by affinity chromatography on C3-Sepharose (Plate 4.6). This indicated that CR1 could be isolated by both methods, however as purification of CR1 on C3-Sepharose was very time consuming it was decided to use the immunoprecipitation technique in all subsequent experiments.

4.26 Stability of E CR1

The stability of CR1 on E was assessed by radiolabelling the E and incubating the cells for 1 hour, 2 hours or 4 hours in PBS at 37°C before lysing the E. Plate 4.7 shows that within a 4 hour incubation period a CR1 doublet of 220,000 daltons and 250,000 daltons could be seen at all times. When the cells were labelled and incubated for 48 hours at 37°C or 4°C in autologous plasma or RPMI/BSA a band of 220,000 daltons could be seen indicating that the receptor had not been shed (Plate 4.8).

Table 4.7 Determination of E CR1 in the Presence of Opsonised IgG Aggregates

| | CR1 Sites/E | | |
|---|---|---|---|
| | Unopsonised IgG | Opsonised IgG | Percentage Change |
| Donor 1 | 4057 | 0 | -100 |
| Donor 2 | 5717 | 1621 | -72 |

Note

CR1 numbers were measured on E in the presence of opsonised and unopsonised IgG aggregates. The change in CR1 levels caused by opsonised IgG aggregates was calculated as a percentage of the CR1 sites per E in the presence of unopsonised IgG aggregates.

Table 4.8 Modulation of E CR1 Numbers in Response to Oral Antigen Challenge

| Interval after Oral Antigen Challenge (min) | Number of Donors | Mean Percentage Change from Control±SD |
|---|---|---|
| 30 | 6 | -5±9 |
| 60 | 6 | + 5±16 |
| 120 | 6 | -33±13 |
| 180 | 6 | -14±7.4 |
| 240 | 6 | -4.6±10 |

Note

CR1 levels were measured on E at various time intervals up to 240 min after ingestion of 1.2 litres of cows milk. The change in E CR1 number at each time interval was calculated as a percentage of the CR1 sites present on the donors E immediately prior to milk consumption (control). The mean percentage change in E CR1 levels was calculated for each group of 6 donors at each time point.

Fig 4.6 Modulation of E CR1 Expression in Response to Oral Antigen Challenge

Legend

Erythrocyte CR1 levels were measured on 6 individuals at various time intervals up to 240 min after ingestion of 1.2 litres of cows milk ( X axis). The change in E CR1 for these 6 donors was calculated as a percentage of the 0 time point ( Y axis). The 0 time point represents the number of CR1 sites per E present immediately prior to milk consumption. The plotted values represent the mean ± SEM for 6 donors.

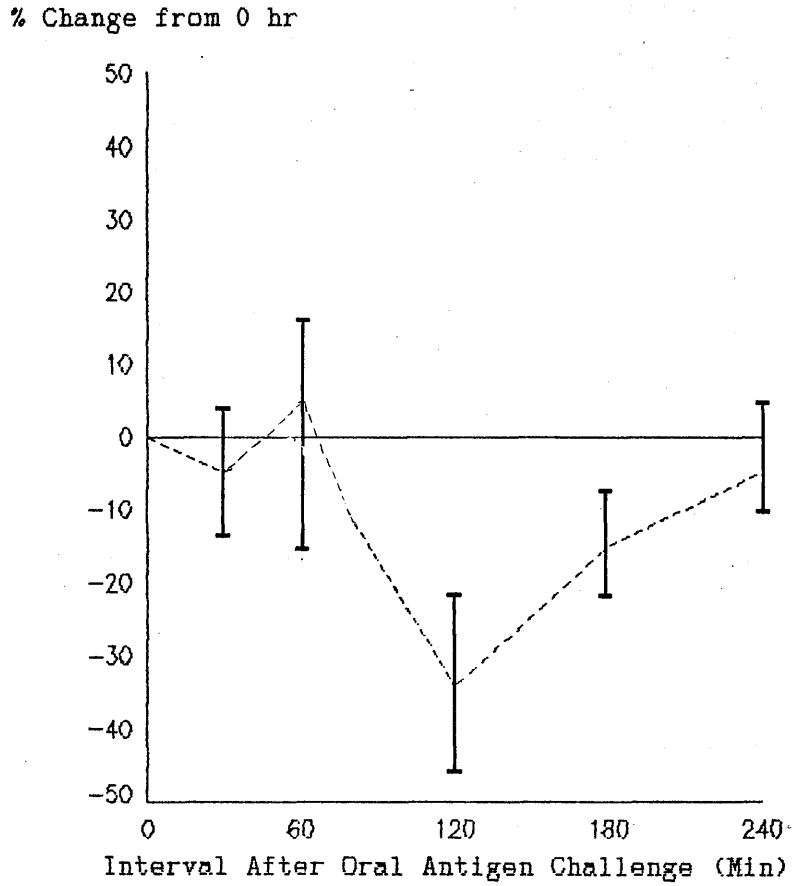

% Change from 0 hr
50
40
30
20
10
0
-10
-20
-30
-40
-50
0
60
120
180
240
Interval After Oral Antigen Challenge (Min)

Table 4.9 Modulation of E CR1 Numbers in Fasting Individuals

| Interval after Oral Antigen Challenge (min) | Number of Donors | Mean Percentage Change from Control |
|---|---|---|
| 60 | 2 | +1.75 |
| 120 | 2 | -5.50 |
| 180 | 2 | -3.50 |

Note

CR1 levels were measured on E at various time intervals following an overnight fast. The change in E CR1 number at each time point was calculated as a percentage of the CR1 sites present on the donor's E when the first blood sample was collected (control). The mean percentage change at each time point was calculated for each 2 donors.

Table 4.10 Incubation of E with Sera Collected from Donors after Ingestion of 1.2 Litres of Cows Milk

| Interval after Ingestion of 1,2 Litres of Milk (min) | Number of Serum Samples Tested | Mean Percentage Change in ECR1 Levels on an $O^{+VE}$ Blood Donor±SE |
|---|---|---|
| 30 | 2 | -1,0±9,2 |
| 60 | 4 | -10,0±9,5 |
| 120 | 4 | +7,25±11 |
| 180 | 4 | +1,25±7,0 |
| 240 | 3 | -3,3±18 |

Note

CR1 number was calculated on the E of an $O^{+VE}$ blood group donor after incubation of their E in the sera from individuals who had ingested 1.2 litres of cows milk. The number of CR1 sites present on E after incubation in sera collected before milk consumption was used as a control and the subsequent measurement of CR1 sites on E which had been incubated in sera collected after milk consumption were expressed as a percentage of this control.

Plate 4.5 Immunoprecipitation of CR1 from Human E

Legend

Immunoprecipitation of CR1 from human E using an anti-CR1 monoclonal antibody (track a) and polyclonal IgG anti-CR1 (neat; track b and diluted 1:10; track c). Samples were analysed on 5% slab gels run under reducing conditions. A band of molecular weight 220,000 daltons is seen in tracks a, b and c. Tracks d and e are controls immunoprecipitated with NMS.

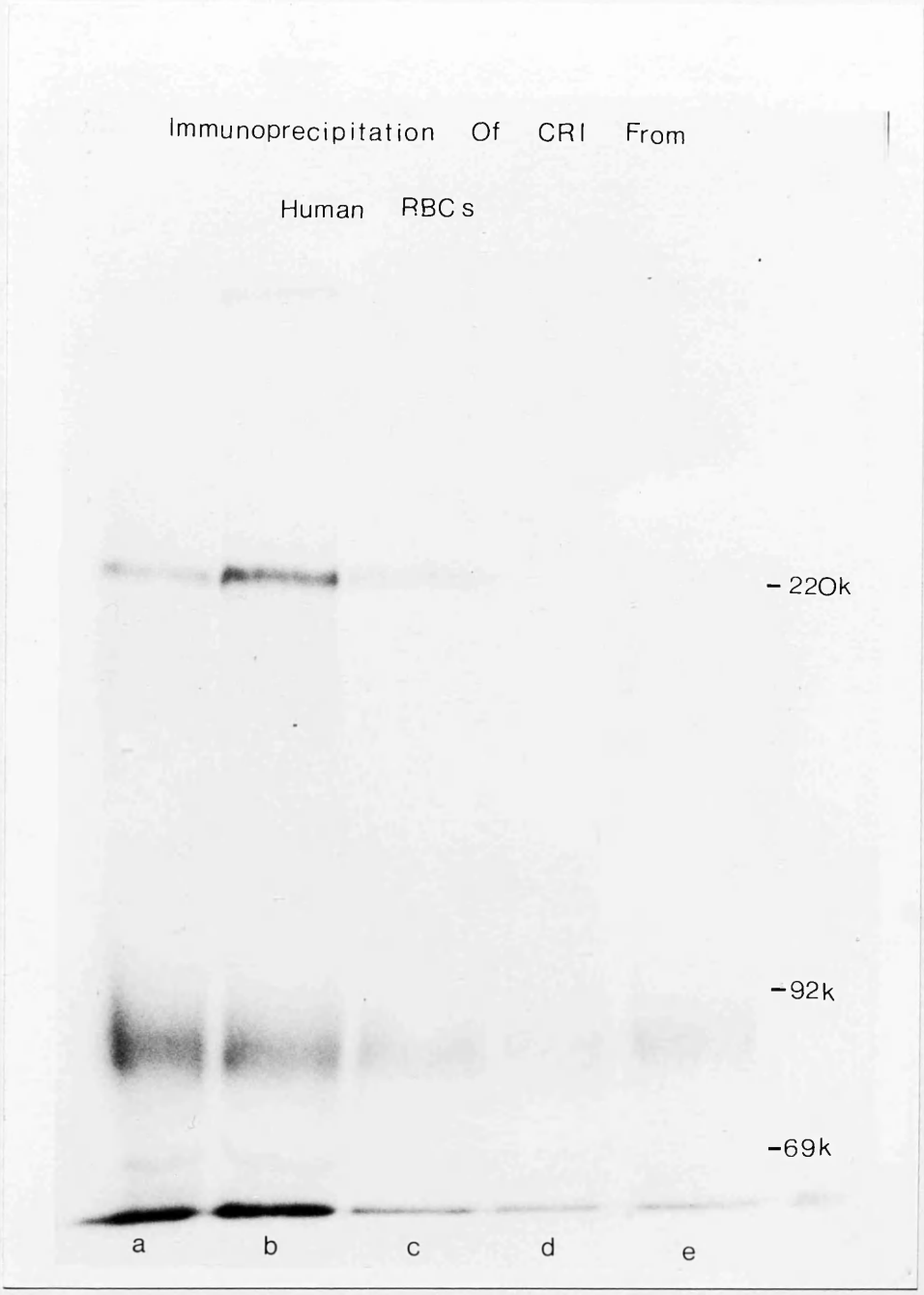

Immunoprecipitation Of CR1 From
Human RBCs
-220k
-92k
-69k
a
b
c
d
e

Plate 4.6 Elution of CR1 from C3-Sepharose

Legend

A band with a molecular weight of 220,000 daltons was separated from solubilised E membranes by affinity chromatography on C3-Sepharose (tracks a and b). No bands were seen in the control samples (tracks c and d) which were eluted from IgG-Sepharose. Samples were analysed on 5% slab gels run under reducing conditions.

Elution Of CR1 From C3—Sepharose

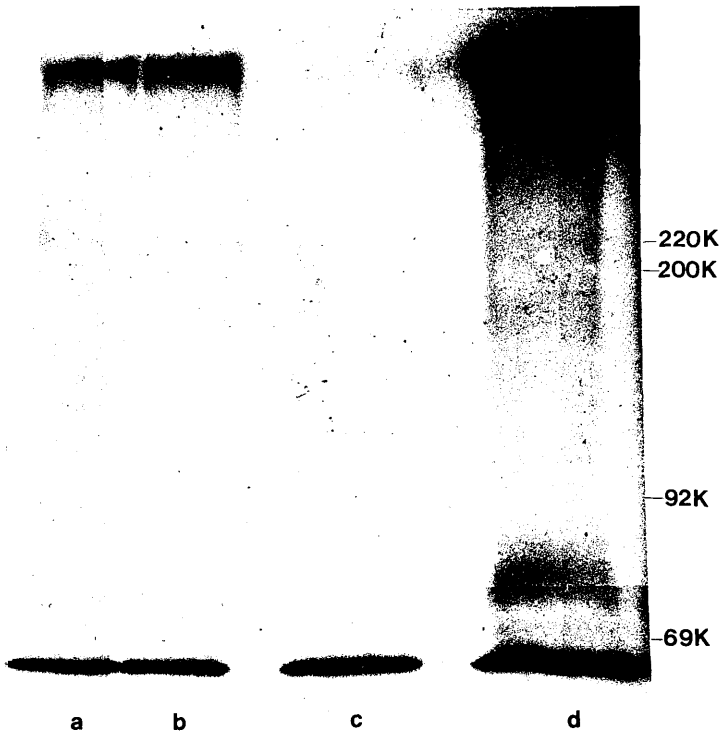


Plate 4.7 Immunoprecipitation of CR1 at Various Time Intervals After Labelling E

Legend

Erythrocytes were labelled with $^{125}I$ and either lysed immediately and immunoprecipitated with anti-CR1 (track a) or incubated for 1 hour (track c), 2 hours (track e) or 4 hours (track g), before being lysed and immunoprecipitated with anti-CR1. Tracks b, d, f and h are controls immunoprecipitated with NMS. When samples were analysed on 5% slab gels run under reducing conditions, 2 bands with molecular weights of 220,000 and 250,000 daltons were immunoprecipitated from E treated with anti-CR1. This represents 1 of the structural polymorphic variants of CR1 (Dykman et al 1983a).

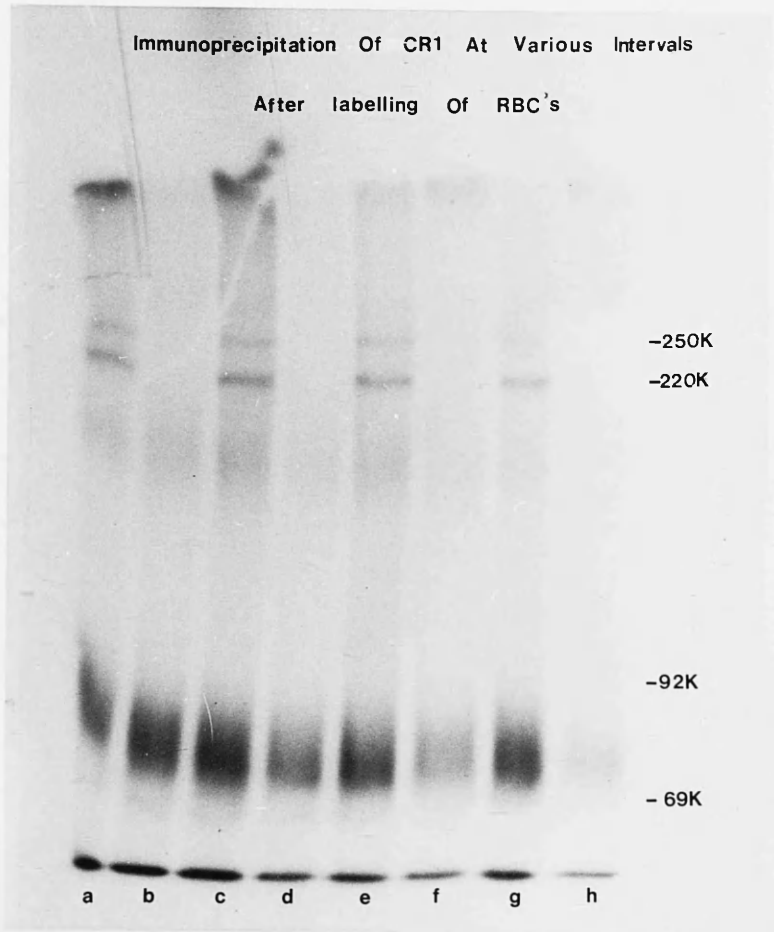
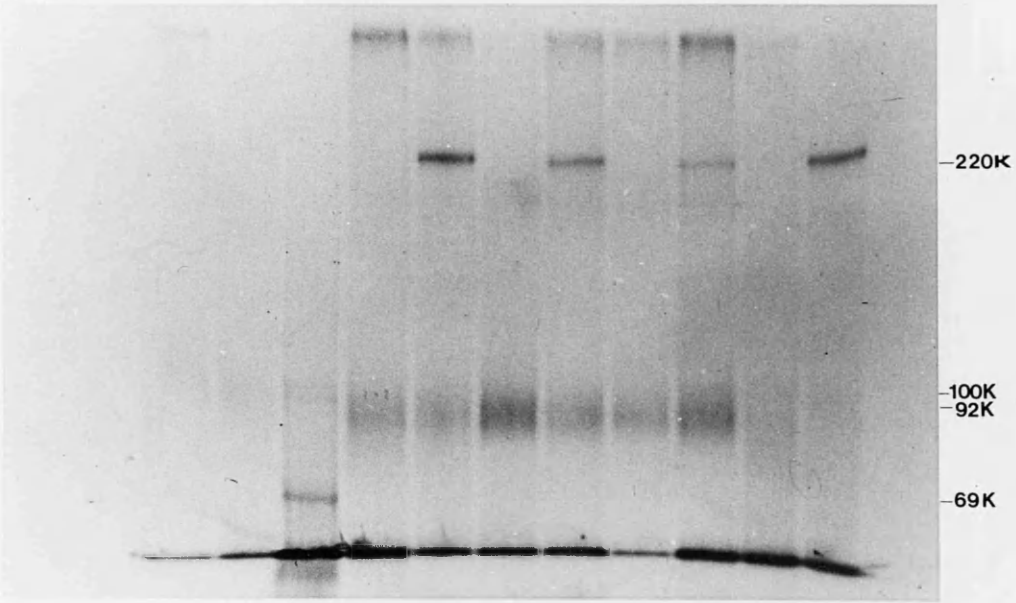

Immunoprecipitation Of CR1 At Various Intervals
After labelling Of RBC's
-250K
-220K
-92K
- 69K
a
b
c
d
e
f
g
h

Plate 4.8 CR1 on E Labelled and Incubated for 48 Hours in Plasma or RPMI

Legend

Erythrocytes were labelled and incubated for 48 hours in RPMI/BSA at 4°C (track b), autologous plasma at 37°C (track d) or autologous plasma at 4°C (track f), before being immunoprecipitated with anti-CR1. Track h shows E with no incubation. Tracks a, c, e and g are controls immunoprecipitated with NMS. Samples were analysed on 5% slab gels run under reducing conditions. A band of molecular weight 220,000 daltons was immunoprecipitated from each of the samples which were treated with anti-CR1. No specific bands were immunoprecipitated with NMS.

CR1 On RBC's Labelled And Incubated For 48hrs In Plasma Or RPMI



4.27 Trypsinisation of E CR1

The same concentrations which were used to study the removal of all CR1 detectable by RIA were used to study removal of CR1 on surface labelled cells. A concentration of 0.01mg/ml was found to be the minimum concentration which removed all CR1 (Plate 4.9). This is the same concentration which removed all CR1 detectable by RIA (Table 4.5).

4.28 Effect of Patients Sera on E CR1

To investigate whether some component of patients serum could cause partial proteolysis of CR1, E from a donor known to have CR1 numbers in the middle of the normal range were radiolabelled and incubated overnight at 37°C in serum from a patient with SLE in the active phase.

As can be seen from Plate 4.10 a band of molecular weight 220,000 daltons representing CR1 was immunoprecipitated from the E after an overnight incubation at 37°C in this serum. There were no other specific bands evident indicating that the SLE serum did not contain any component which altered CR1 structure. Radiolabelled E were also incubated overnight at 37°C in the serum from 2 patients with RA, 1 in an active state of disease and 1 in an inactive state of disease. Patients with RA have been shown to have slightly reduced E CR1 levels as compared with normal individuals (Ross et al 1985; Holme et al 1986).

Plate 4.11 (tracks a, c and e) shows that a band of 220,000 daltons could be immunoprecipitated from the E in each case. No other specific bands were seen indicating that the CR1 structure had not been altered.

The cells from the donor used in this experiment were also treated with 0.01mg/ml of trypsin which removed all CR1. After removal of CR1 the E were not surface labelled but incubated overnight at 37°C in PBS or sera from RA patients in active and inactive states of disease to see if reconstitution of CR1 occured after removal of CR1 by trypsin. No regeneration of the receptor was found on the E incubated in any type of medium (Plate 4.11 tracks f, h and i). Even when trypsinised E were incubated for 3 days at 37°C in autologous plasma, PBS or RPMI/BSA no regeneration of CR1 occurred (results not shown).

Plate 4.9 Effect of Varying Concentrations of Trypsin on Expression of CR1 on E

Legend

Erythrocytes were trypsinised with various concentrations of trypsin, 1mg/ml (track c), 0.01mg/ml (track e) and 0.001mg/ml (track g), radiolabelled and immunoprecipitated with anti-CR1. Track a shows untrypsinised E and tracks b, f and h are the controls immunoprecipitated with NMS. Samples were analysed on 5% slab gels run under reducing conditions. A band with a molecular weight of 220,000 daltons was immunoprecipitated with anti-CR1 from untrypsinised E (track a) and E trypsinised with 0.001mg/ml of trypsin.

Effect Of Varying Concentrations Of Trypsin On Expression Of CR1 On RBC's

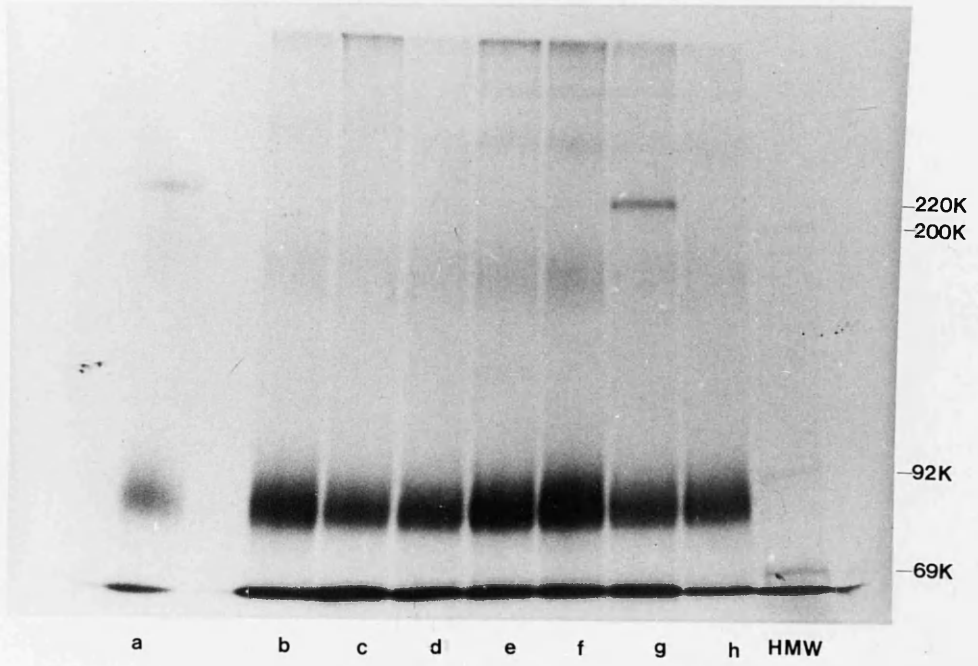


Plate 4.10 Effect of SLE Serum on E CR1

Legend

E were radiolabelled and incubated overnight at 37°C in serum from a patient with SLE in an active phase. The E were then lysed and immunoprecipitated with anti-CR1 (track b) and NMS (track a). The samples were analysed on a 5% slab gel run under reducing conditions. A specific band with a molecular weight of 220,000 daltons, representing CR1, was seen on the E immunoprecipitated with anti-CR1 (track b). No other specific bands were seen.

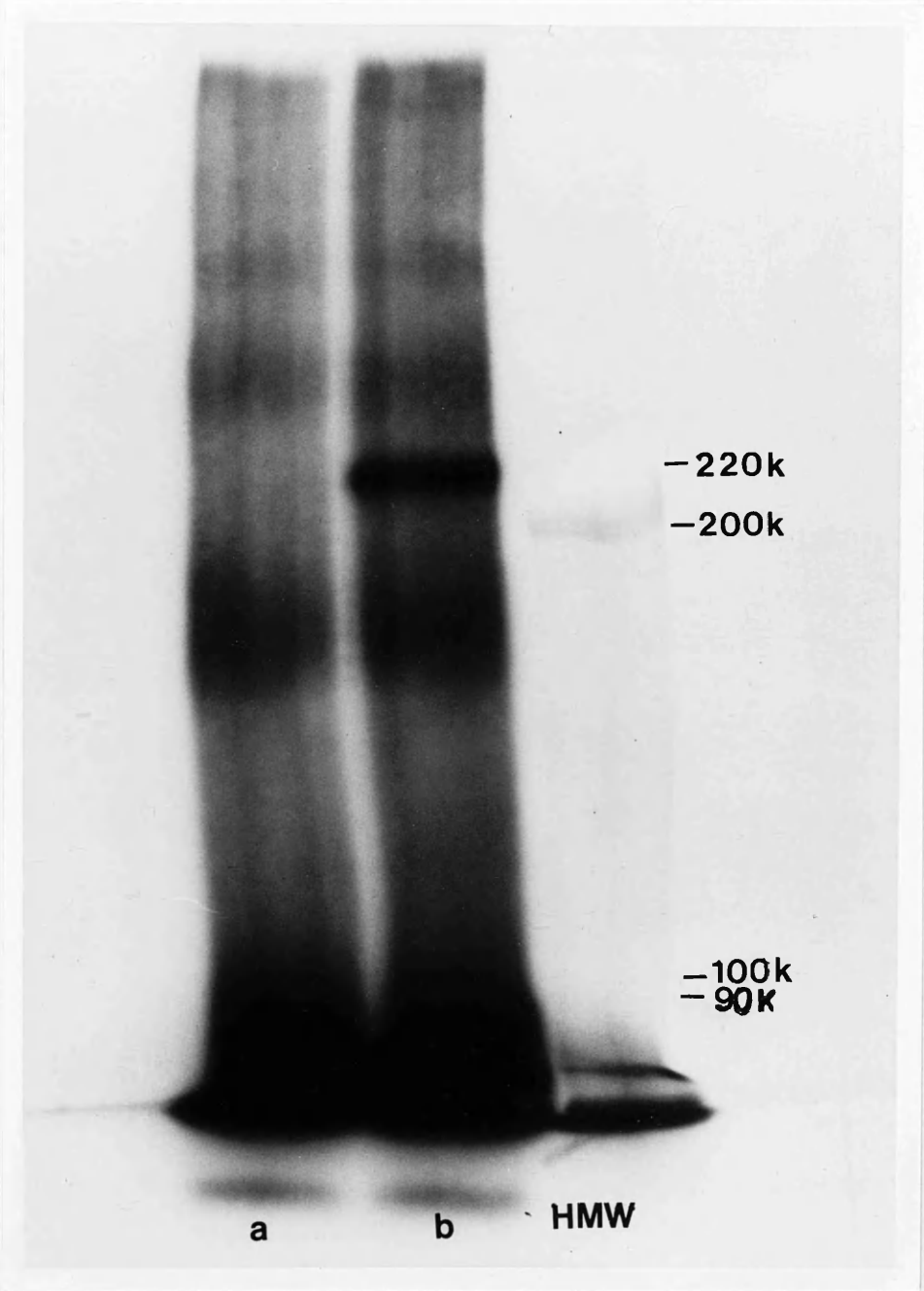

—220k
—200k
—100k
—90k
a
b
HMW

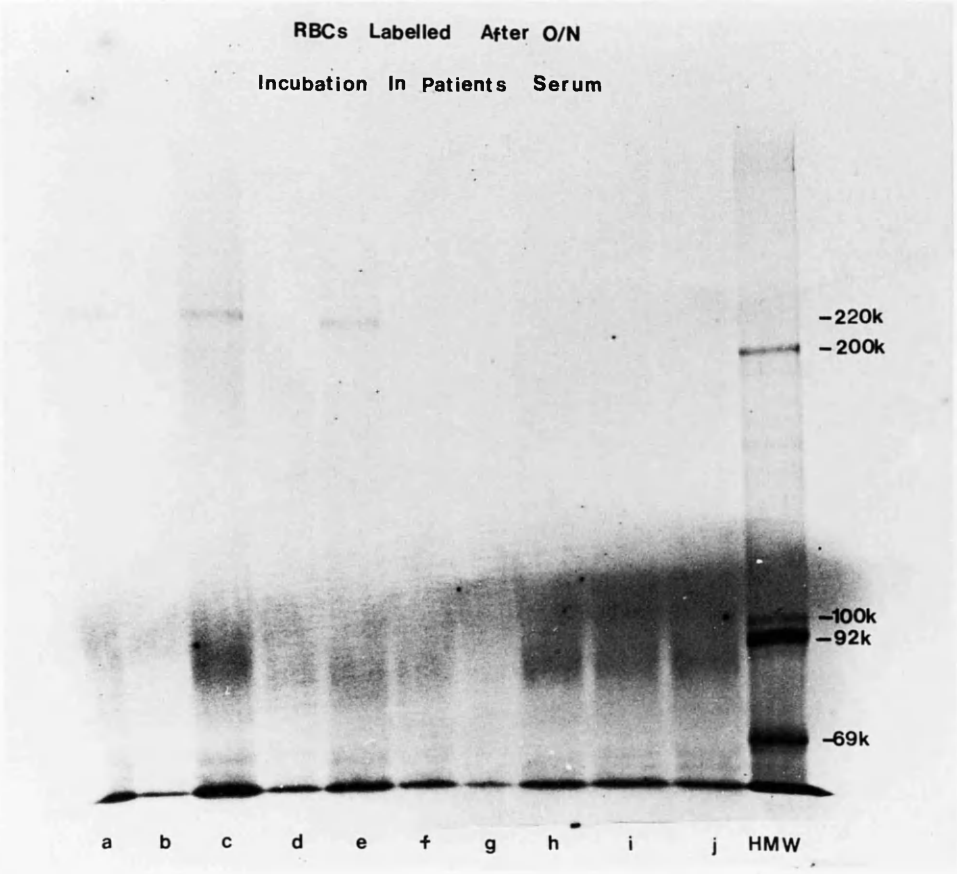
Plate 4.11 Effect of RA Serum on E CR1

Legend

Erythrocytes were radiolabelled and incubated overnight at 37°C in PBS (track a) or serum from a RA patient whose disease was in remission (track c) or serum from a RA patient whose disease was in an active phase (track e). The E were then lysed and immunoprecipitated with anti-CR1

Erythrocytes from the same donor were treated with 0.01mg/ml of trypsin and incubated overnight in PBS (track f) or in sera from RA patients in an inactive (track h) or an active (track i) state of disease. These E were then lysed and immunoprecipitated with anti-CR1. Tracks b, d, g and j are controls immunoprecipitated with NMS.

All samples were analysed by SDS-PAGE (5% slab gels) run under reducing conditions. A band with a molecular weight of 220,000 daltons was seen on E immunoprecipitated with anti-CR1 after incubation overnight in PBS or in sera from RA patients (tracks a, c and e). No specific bands were immunoprecipitated from trypsinised E (tracks f, h and i).


RBCs Labelled After O/N
Incubation In Patients Serum
-220k
-200k
-100k
-92k
-69k
a
b
c
d
e
f
g
h
i
j
HMW

## 4.29 Discussion

The results of the twin study showed that in an normal population E CR1 were regulated by environmental factors. The experiments described in this chapter were performed to investigate possible mechanisms which may account for the reduced E CR1 expression which is seen in patients with SLE. Three possibilities were examined, (i) CR1 stability, (ii) CR1 sensitivity to proteolytic enzymes, and (iii) CR1 blockade by opsonised immune complexes.

The 2 methods selected to investigate these possibilities were (i) quantification of E CR1 using a polyclonal or monoclonal antiserum and (ii) surface labelling of E CR1 and analysis of the receptor visually by autoradiography.

Using the polyclonal antiserum a mean of 3,773 CR1 sites/E was obtained. This is within the normal range of that reported by Holme et al (1986) using the same polyclonal antiserum (range 335-8,009). The polyclonal antiserum was used in some instances as supplies of the monoclonal antibody E11 were limited.

Surface labelling of E and immunoprecipitation of CR1 with a monoclonal anti-CR1 antiserum resulted in the precipitation of 2 polymorphic forms of CR1. One with a major band of molecular weight 220,00 daltons (Plate 4.5) and 1 with 2 major bands of molecular weight 220,000 and 250,000 daltons (Plate 4.7). The 250,00 dalton band represents a less common phenotypic variant of CR1 and the individual from whom these E were obtained is heterozygous expressing 1 allele for the common CR1 form and 1 for the less common higher molecular weight variant. These bands are 30,000 daltons higher than the bands reported by Dykman et al (1983(a)) who ran gels under non-reducing conditions. In reduced gels the estimated molecular weight of CR1 is 30,000 daltons greater than that which is estimated in gels run under non-reducing conditions. In some instances a less intense band was seen which was 15,000 daltons higher than the major band (Plate 4.11). This is similar to a band described by Dykman et al (1983(a)). However, the nature of this band is unknown. CR1 was also successfully isolated from radiolabelled E by C3-Sepharose affinity chromatography (Plate 4.6).

Having established that the 2 methods were valid for detecting E CR1, the stability of the receptor was investigated. Storage overnight at 4°C or 37°C in RPMI/BSA or autologous serum did not result in a significant reduction in CR1 sites/E (Table 4.2; Table 4.4). This conclusion was supported by the finding that CR1 could be immunoprecipitated successfully from surface labelled E after incubation for 2 days at 37°C in autologous plasma (Plate 4.8). Thus in normal individuals it would appear that shedding of E CR1 is unlikely. It has been reported that E with low CR1 activity show high osmotic fragility (Inada et al 1983a) and it is known that anaemia is a common manifestation in SLE appearing in approximately 80% of patients. Thus it is possible that alterations in

the membrane physiology of the E of patients with SLE does result in a spontaneous release of CR1. In this respect it would be interesting to measure the serum concentrations of the soluble form of CR1 to see whether they are higher in patients with SLE in an active phase than they are in normal individuals (Yoon and Fearon 1985) or in patients with SLE in remission.

The removal of CR1 by proteases is at present thought to be the most likely explanation for reduced E CR1 numbers (Ross et al 1985; Ripoche and Sim 1986). Proteolytic enzymes such as thrombin, plasmin and kallikrein normally circulate as inactive precursors and not in their active form. However when tissue damage occurs such as in immune complex disease, activation of the plasma mediator systems occurs so that plasma, thrombin and kallikrein are present in their active form for short periods. It is possible that these enzymes could proteolytically strip CR1 from the surface of E membranes.

Experiments were performed to determine if these proteases could remove CR1 in vitro. It was found that at the physiological concentrations used thrombin, plasmin and kallikrein did not significantly reduce E CR1 numbers (Table 4.5). This is in contrast to the findings of Ripoche and Sim (1986) which showed that in vitro CR1 was sensitive to physiological concentrations of plasmin (1.56-100$\mu$g/ml) and thrombin (1.25-20 units/ml). At these concentrations CR1 was removed from E as assessed by the appearance of a form of CR1 in the supernatant which had a cofactor activity for the I mediated cleavage of C3b to iC3b. However they could not demonstrate the loss of CR1 from E during clotting in whole blood which is an indication that during clotting in vitro there is an insufficient amount of free thrombin and plasmin generated to cause removal of CR1 from E.

The leukocyte proteases cathepsins C and D were also included in the study but they were not found to have any effect on CR1 (Table 4.5).

The trypsin sensitivity of CR1 has been well documented (Nelson and Nelson 1959; Sim 1985; Ripoche and Sim 1986) and in this study I have shown that trypsin could remove CR1 from E at a concentration of 0.01mg/ml. Elastase, a protease secreted by leukocytes also caused removal of CR1 when it was used at a high concentration (Table 4.5). It is unlikely that the amount of elastase or trypsin in the serum would be sufficient to remove CR1 as the serum protease inhibitors $\alpha 2$ - macroglobulin and $\alpha$ 1-anti-trypsin are present in serum in such high concentrations that they would inactivate these enzymes. However if E were in intimate contact with phagocytic leukocytes such as is the case when E carrying immune complexes pass through the liver and the spleen (Cornacoff et al 1983) removal of CR1 may occur randomly through contact with the proteinase-rich tissue phagocytes. In patients with SLE the extent and duration of contact between E and tissue phagocytes might increase due to the elevated levels of immune complexes present on the E surface.

The experiments with trypsin also indicated that once CR1 was removed no new receptors could be brought to the cell surface (Plate 4.11). This indicates that there is no obvious internal pool of CR1 or that if one does exist, that under the in vitro conditions used, the E were unable to mobilise the pool perhaps due to membrane damage caused by trypsin. Sim and Sim (1983) also concluded that there was no internal pool of CR1 in E after the observation that the cofactor activity of CR1 for the I mediated cleavage of C3b on intact E was comparable to that of soluble E, indicating that all of the E CR1 was expressed on the cell surface.

Treatment of soluble CR1 with trypsin results in its breakdown to disulphide-linked fragments with molecular weights of 65,000 and 160,000 daltons. These fragments are subsequently degraded to lower molecular forms upon prolonged treatment with trypsin (Sim 1985). CR1 was immunoprecipitated from E after an overnight incubation at 37°C in serum from patients with active SLE (Plate 4.10) and active RA (Plate 4.11). No lower molecular weight degradation fragments of CR1 were observed indicating that these sera did not contain active proteases.

The final aspect which was looked at was the ability of the receptors to be blocked by immune complexes. It has been documented that E CR1 is not a stable phenotype in patients with SLE as the CR1 levels on the E of these patients can increase or decrease as a patient enters an active or inactive state of disease. Ross et al (1985) and Holme et al (1986) have shown that when a patient enters remission, E CR1 numbers increase within a time interval which is incompatable with entry of newly synthesised E into the blood stream. Thus the possibility exists that complexes may have been blocking the access of radiolabelled probes to the receptor when the disease was in its active phase with high levels of circulating immune complexes and as the patient went into remission that the complexes were removed leaving the CR1 binding site free. In favour of this hypothesis Inada et al (1982) observed that the E from SLE patients showed defective CR1 activity when immune complexes were present in the serum and Wilson et al (1985b) found that high titres of an autoantibody to CR1 were correlated with the absence of CR1 from E and heightened disease activity in 1 SLE patient.

However Wilson et al (1982), Minota et al (1983) and Ross et al (1985) published findings which were inconsistent with blockade of existing receptors by immune complexes. Ross et al (1985) failed to find evidence that E from SLE patients contained abnormal amounts of C3 antigen an indication that E did not contain surface bound C3b or iC3b and therefore could not contain CR1 bound complexes which required either C3b or iC3b for attachment. CR1 sites could also be detected with $^{125}$I-E11 even when E had bound dsDNA/anti-DNA complexes. However the concentration of complexes may have been too small to cause blockade of CR1 sites. Experiments in our laboratory by Dr E Holme suggest that the size of immune complex is crucial as E CR1 numbers were unchanged by small complexes but could be blocked completely when large complexes were

bound. This was probably as a result of steric hindrance between the complexes and $^{125}$I-E11 which was used as the probe.

The results of an in vivo and an in vitro experiment are presented in this chapter. Both suggest that blockade of CR1 by complexes could occur. In vitro it was shown that CR1 number on the E of 2 individuals were considerably reduced in the presence of large opsonised IgG aggregates as compared to the levels in the presence of large unopsonised complexes (Table 4.7).

Food consumption is one aspect of daily living which results in the formation of immune complexes containing food antigens (Paganelli et al 1979). It was therefore decided to study CR1 levels on the E of individuals after they had consumed 1.2 litres of cows milk which is an easily absorbed rich source of food antigens to see if in vivo formation of complexes would reduce E CR1 levels. It was shown that 2 hours after milk consumption E CR1 levels were reduced and by 4 hours they had returned to normal (Table 4.8). The E used in control experiments where no milk was consumed showed no change in E CR1 number (Table 4.9).

To test for the presence of complexes containing food antigens in the sera from individuals after milk consumption the sera were incubated with E from donors with an $O^{+ve}$ blood group. The results showed that there was no significant change in E CR1 numbers caused by any of the sera (Table 4.10). This does not necessarily imply that the sera did not contain complexes. The complexes may have been in a form which were unable to rebind E CR1 as a result of processing of C3b into smaller fragments such as C3dg which is not a ligand for CR1.

Time did not permit further investigation of this phenomenon. Future studies could look for the presence of C3 and IgG on the surface of E after milk consumption and also for the presence of complexes containing the milk antigens caesin and lactalbumin on the surface of E and in the serum. Even if these studies could not show the presence of complexes on the cells the results from the experiment indicate that some mechanism must exist which allows rapid increases and decreases in E CR1 numbers which can definitely not be accounted for by proteolysis.

Thus the situation would appear to be complex and at the present moment it is not possible to determine if blockade by complexes or enzymatic removal of CR1 is the cause for reduced E CR1 numbers in E SLE. The fact that serial studies show that E CR1 levels can return to normal before new E can be produced (Ross et al 1985, Holme et al 1986) argues against the theory that proteolytic stripping of CR1 is the mechanism; however it cannot be excluded that proteolysis contributes towards the extent of reduced CR1 expression in SLE. The results presented in this chapter indicate that CR1 levels can be modulated by environmental factors such as the presence of high concentrations of proteases or the presence of large immune complexes. They also lend support to the theory that E CR1 numbers are not a stable heritable characteristic but that

they may be strongly influenced by simple environmental effects such as eating a meal.

# CHAPTER FIVE

## 5.1 Introduction

In the previous chapter I discussed mechanisms whereby E CR1 levels may be reduced in patients with SLE. There are few published studies of CR1 numbers on the peripheral blood leukocytes of patients with SLE. Such studies are important as leukocytes play a major role in host defence and thus leukocyte CR1 deficiency might produce serious impairment of the ability of the individual to combat infection. As discussed in the introduction CR1 plays a number of important roles on phagocytic cells (1.28). It has the ability to promote endocytosis of small immune complexes (Abrahamson and Fearon 1983) and when CR1 becomes activated it can directly mediate the phagocytosis of large particles such as E and bacteria (Griffin and Griffin 1979; Wright and Silverstein 1982; Wright et al 1983a; Pommier et al 1984) Phosphorylation of CR1 on phagocytes may account for the transition of CR1 from an inactive to an active phagocytic state (Changelian and Fearon 1985)).

In order to gain an understanding of possible defects in CR1 levels on the PMN of patients with SLE it was first of all important to define more closely the distribution of CR1 on normal PMN. CR1 levels on PMN have been quantified previously using both polyclonal and monoclonal anti-CR1 antisera. Fearon (1980) reported that PMN expressed approximately 57,000 binding sites for CR1 using a polyclonal antiserum, whilst Hogg et al (1984) reported 46,000 using the monoclonal E11. Early studies with rosettes revealed that the number of CR1 on the surface of PMN could be increased significantly by exposing the cells to chemotactic stimuli such C5a, histamine or FMLP (Anwar and Kay 1978; Kay et al 1979). It is now known that CR1 numbers on PMN will spontaneously increase when PMN are warmed to 37°C (Fearon and Collins 1983) and increase a further 2- to 3-fold when stimulated with FMLP (Berger et al 1984; Richerson et al 1985) or with low concentrations of phrobolmyristate acetate (PMA), a tumour promoting phorbolester (Changelian et al 1985). This increase in CR1 number is not accompanied by an increase in total cellular CR1 indicating that the additional receptors are being translocated from an internal pool (Changelian et al 1985; O'Shea et al 1985a).

In this chapter I will describe attempts which were made to establish how many pools of CR1 were contained within PMN. The cytoskeletal and energy requirements for expression of these pools were also investigated.

This was achieved in 2 ways, (i) removing the receptors from the surface membrane by trypsinisation and then quantifying the receptors which were regenerated and (ii) treating PMN with FMLP in order to allow maximum expression of CR1. In addition various drugs which cause inhibition of protein synthesis or inhibition of energy production or disruption of cytoskeleton assembly allowed the mechanisms which control the translocation of intracellular CR1 to the surface to be investigated.

Having defined the number of CR1 pools and the conditions under which they were expressed it was then possible to study CR1 on the PMN of patients with SLE to determine if there were any major differences.

Material and Methods

## 5.2 Chemicals and Reagents

Chemicals and other reagents were obtained from the following companies:

Sterling Research, Onslow Street, Guilford, Surrey, England

Hypaque (Sodium Diatrizoate injection BP) 45%

Pharmacia House, Midsummer Boulevard, Milton Keynes, England

Ficoll

Sigma Chemical Co, Fancy Road, Poole, Dorset

Colchicine
Cycloheximide
Cytochalasin B
Dextran
Dinitrophenol (DNP)
N-Formyl-Methionyl-Leucylphenylalanine (FMLP)
Leupeptin
Lummicolchicine
Pepstatin A
Puromycin
Soybean Trypsin Inhibitor (SBTI)
Trypan Blue
Trypsin

Blood Transfusion Service, Law Hospital, Carluke, Scotland

AB plasma pack

Millipore (UK) Limited, Peterborough Road, Harrow, Middlesex

Sterile Millipore Filters

Gibco Biocult, Paisley, Renfrewshire, Scotland

Hepes Buffered Earles Minimal Essential Medium (HEM)

## 5.3 Isolation of PMN from whole Blood

### Buffers and Solutions

#### Ficoll-Hypaque

Ficoll-Hypaque (SG 1.077) was prepared by mixing 20mls of 45% Hypaque with 6.5mls of deionised water to achieve a 33.9% solution. After removal of 1.5mls of this solution 60mls of 9%(w/v) Ficoll were added.

#### Normal Saline

Normal saline was prepared as described in chapter 2.7(b).

#### Dextran Solution

Dextran solution was prepared by mixing 6g of dextran in 100mls of normal saline.

#### Twice Normal Saline (2N Saline)

Twice normal saline (0.3 mol $l^{-1}$) was prepared by mixing 1.8g of sodium chloride in 100mls of deionised water.

#### RPMI/BSA/EDTA

RPMI-1640 containing 0.5%(w/v) BSA and EDTA (0.02 mol $l^{-1}$)

### Procedure

Sixty millilitres of fresh venous blood were transferred to 3 universal containers containing 100µl of sodium heparin(1000 units/ml) to prevent clotting. The blood was then layered onto 15mls of Ficoll-Hypaque in 2 sterile Falcon tubes. The tubes were centrifuged at 400g for 30 min at room temperature. The mononuclear cells were removed and the remaining blood was mixed with an equal volume of autologous plasma, which had been removed from the top of the Ficoll-Hypaque column, and allowed to sediment at 37°C in the presence of 6%(w/v) dextran. The PMN enriched supernatant was removed and the cells pelleted by centrifugation at 200g for 5 min at 4°C. Contaminating E were lysed by resuspending the cell pellet in deionised water for 10 seconds followed by the addition of an equal volume of 2N saline to restore isotonic conditions. The cells were centrifuged at 200g for 5 min at 4°C and the lysed E removed with a Pasteur pipette. The PMN were resuspended in 20mls of RPMI/BSA/EDTA, 10µl of this suspension were diluted with 180µl of white cell counting fluid and the number of cells counted using an improved Neubauer haemocytometer. The PMN were then resuspended in RPMI/BSA/EDTA at $5x10^6$/ml.

## 5.4 Enumeration of PMN CR1 Numbers with $F(ab')_2$ anti-CR1

### Reagents

### Pepstatin A

Pepstatin A was stored at -20°C at 1mg/ml in DMSO.

### Leupeptin

Leupeptin was stored at -20°C at 1mg/ml in deionised water.

### Buffers

### RPMI/BSA/EDTA/I

0.1%(w/v) soybean trypsin inhibitor (SBTI), 25ug/ml pepstatin A and 25ug/ml leupeptin were added to a volume of RPMI/BSA/EDTA. These inhibitors were added to prevent proteolytic degradation of CR1 by enzymes released from the PMN during the assay.

### Procedure

Triplicate aliquots (200ul) of PMN ($5x10^7$/ml) in RPMI/BSA/EDTA/I were transferred to a series of 8 microcaps. Unlabelled nonimmune rabbit $F(ab')_2$ fragments (12.5ug/ml) were added to tubes 1 to 4 and unlabelled anti-CR1 $F(ab')_2$ fragments (12.5mg/ml) were added to tubes 5 to 8. All of these additions were made while the microcap tubes were being incubated on ice. The cells were then mixed on a Matburn mixer at 4°C for 30 min after which incremental quantities (1ug,2ug,3ug,and 4ug) of $^{125}I$-$F(ab')_2$ anti-CR1 were added to each set of 4 microcap tubes. Again these additions were performed on ice. The cells were mixed on a Matburn mixer for 1 hour at 4°C after which each sample was layered over 300ul of dibutylphthalate/dinonylphthalate in microcap tubes and centrifuged at 10,000g for 2 min at room temperature. The cell pellets were cut from the microcap tubes as before and counted in an automatic $\gamma$ counter. This procedure for measuring PMN CR1 numbers is essentially the same as that adapted for measuring E CR1 numbers there are however 2 major differences. Firstly, all additions to the PMN were made on ice, this prevented internalisation of the $F(ab')_2$ anti-CR1 fragments. Secondly, the first incubation time was shortened from 1 hour to 30 min to lessen the chance of CR1 being degraded by PMN proteolytic enzymes or $F(ab')_2$ anti-CR1 fragments being internalised.

### Calculation of CR1 Sites per PMN

Total binding and nonspecific binding curves were constructed as previously described in chapter 4.6(b) and a linear regression analysis

performed. The number obtained from this analysis was converted into CR1 sites per PMN according to the following formula:

The number of molecules in 1cpm (N) =

$$\frac{6.02\times10^{12}}{\text{cpm in 1ug of F(ab')}_2\text{ anti-CR1}}$$

The number of CR1 molecules/PMN =

$$\frac{\text{N x the number of bound cpm in } 1\times10^{6}}{1\times10^{6}\text{(number of cells/microcap tube)}}$$

Intra-Assay Variation

Reproducibility of the assay was assessed by calculating the CR1 number on 3 separate aliquots of an individual blood sample all at the same time. The mean of the 3 numbers obtained in this manner was calculated (Table 5.1). The intra-assay variation was ± 9%.

## 5.5 Measurement of PMN CR1 Numbers using the Monoclonal Antibody E11

Triplicate aliquots (200ul) of PMN ($5\times10^6$/ml) in RPMI/BSA/EDTA/I were mixed on a Matburn mixer with incremental quantities of $^{125}$I-E11 (0.07, 0.7 and 1.4μg) for 1 hour at 4°C. Nonspecific binding was assessed by firstly incubating triplicate aliquots of PMN ($5\times10^6$/ml) with a 10-fold molar excess of unlabelled E11 for 30 min at 4°C before addition of 0.7μg of $^{125}$I-E11. As with the polyclonal anti-CR1 all additions to the cells were made on ice. The cells were layered over dibutylphthalate/ dinonylphthalate in microcap tubes, centrifuged at 10,000g for 2 min at room temperature and the cell pellets collected and counted in an automatic γ counter.

Calculation of CR1 Sites per PMN

The amount of radioactivity bound in the absence of cold E11 represents the total amount of $^{125}$I-E11 bound to the PMN. The amount bound in the presence of 0.7 μg of cold E11 represents the nonspecific binding of $^{125}$I-E11. The nonspecific binding of $^{125}$I-E11 increased in a linear fashion (Table 5.2) and therefore to conserve supplies of E11 which were limited, the nonspecific cpm obtained from this 1 point were doubled and plotted against the total input in order to draw a line representing nonspecific binding of $^{125}$I-E11 (Fig 5.1(a)). The total amount of $^{125}$I-E11 bound was then plotted against the total input of $^{125}$I-E11 (Fig 5.1(a)). From this data a Scatchard plot was constructed as previously

Table 5.1 Intra-Assay Variation

CR1 Sites/PMN

| | First Measurement | Second Measurement | Third Measurement | Mean±SD | % Variation from mean | |
|---|---|---|---|---|---|---|
| Donor 1 | 57,566 | 58,637 | 53,363 | 56,522±2,787 | -4 | +4 |
| Donor 2 | 158,468 | 155,271 | 178,373 | 164,037±12517 | -9 | +5 |

Note: The mean of the 3 measurements was calculated and the percentage variation of the highest and lowest measurement from the mean was calculated.

Table 5.2 Nonspecific Binding of $^{125}$I-E11

| Bound cpm to 1x10$^6$ PMN incubated with 7μg of cold E11 | | | |
|---|---|---|---|
| Input of 125I-11(μg) | Donor 1 | Donor 2 | Donor 3 |
| 0.7 | 386 | 331 | 319 |
| 1.4 | 666 | 710 | 620 |

Fig 5.1(a) Binding of $^{125}$I-E11 to PMN

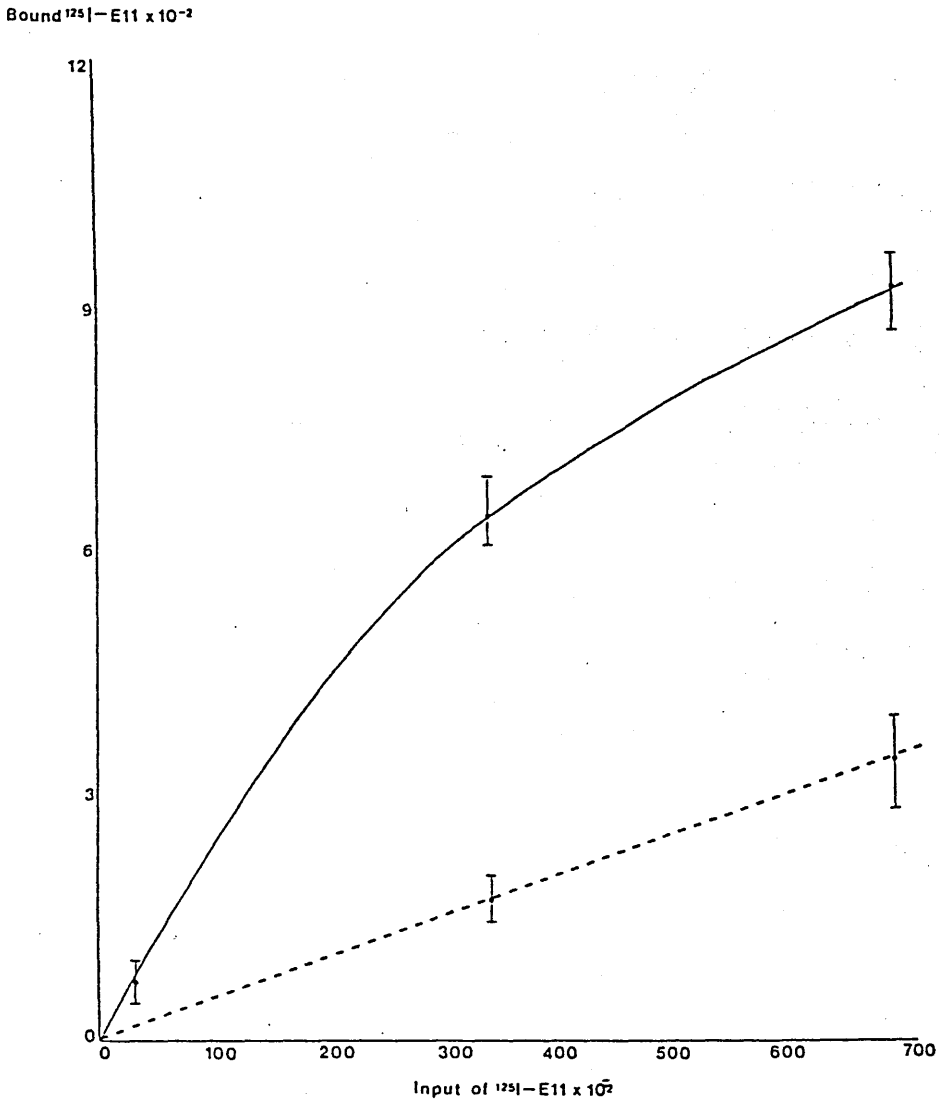
Legend

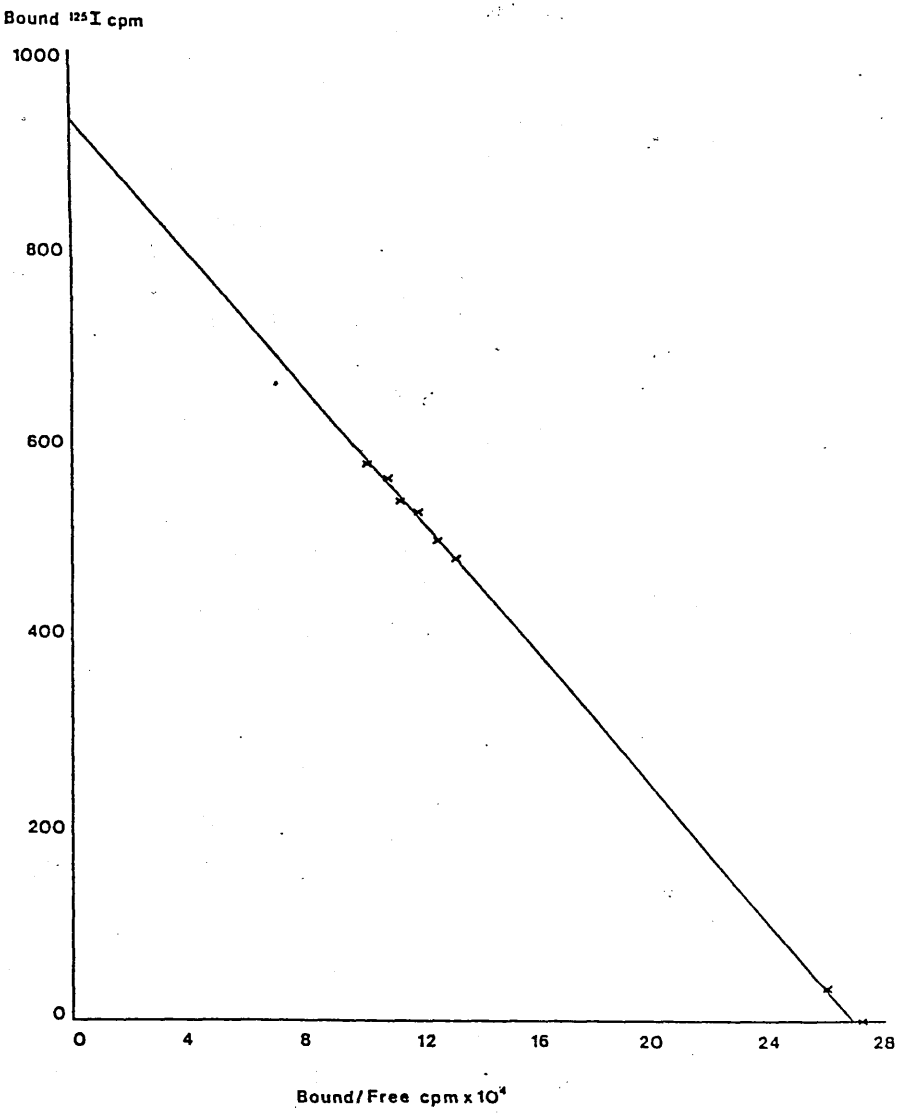
The amount of radioactivity bound to PMN in the presence of incremental quantities of $^{125}$I-E11 (0.07, 0.7 and 1.4$\mu$g) and the amount bound in the presence of a 10-fold molar excess of unlabelled E11 were plotted against the input of E11, with each point being performed in triplicate (the vertical bars represent the SEM). The binding curves show the total amount of $^{125}$I-E11 bound (_____) and the nonspecific binding of $^{125}$I-E11 (- - -). The ordinate axis shows the total input of $^{125}$I-E11 and the abscissa shows the total amount of $^{125}$I-E11 which was bound expressed as cpmx$10^{-2}$.

Fig 5.1(b) Scatchard Plot

Legend

A Scatchard plot was constructed by plotting the number of specific bound $^{125}$I-E11 against the bound/free $^{125}$I-E11 cpm. The point where this line crosses the Y axis, represents the number of bound cpm to 1x$10^{6}$ PMN.


Bound $^{125}$I−E11 x $10^{-2}$
12
9
6
3
0
0
100
200
300
400
500
600
700
Input of $^{125}$I−E11 x $10^{2}$


Bound $^{125}$I cpm
1000
800
600
400
200
0
0
4
8
12
16
20
24
28
Bound/Free cpm x $10^{4}$

described in chapter 4.6(b) (Fig 5.1(b)). The point where this line crossed the Y axis was determined by linear regression analysis with this point representing the number of bound cpm to $1x10^6$ PMN. This number was converted into CR1 sites per PMN according to the formula shown below.

The number of molecules in 1cpm (N) =

$$\frac{2.81x10^{12} \text{ (number of molecules in } 0.7\mu g)}{\text{cpm in } 0.7\mu g \text{ of } F(ab')_2 \text{ anti-CR1}}$$

The number of molecules per PMN =

$$\frac{N \text{ x the number of bound cpm in } 1x10^6 \text{ cells}}{1x10^6 \text{ (number of cells per microcap tube)}}$$

Intra-Assay Variation

The reproducibility of the assay using the monoclonal antibody was assessed by calculating the CR1 number on 3 separate aliquots of an individual blood sample all at the same time (Table 5.3) The intra-assay variation was ±8%.

## 5.6 Comparison of Polyclonal Antiserum with Monoclonal Antiserum

When binding of the polyclonal $F(ab')_2$ anti-CR1 antibody to PMN of 3 normal donors was compared with that of monoclonal anti-CR1 antibody a mean of 2.2 molecules of $F(ab')_2$ polyclonal antibody was bound per binding site for monoclonal antibody E11. Thus it is possible to compare results obtained by both methods.

## 5.7 Study of PMN CR1 Number Changes in Response to Drugs

### Reagents

N-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP)

FMLP a synthetic chemotactic peptide which causes chemotaxis of PMN and other phagocytic cells, was stored at -70°C as a $10^{-2}$ mol $l^{-1}$ stock solution made up in dimethyl sulphoxide (DMSO). This stock solution was diluted to a final concentration of $10^{-6}$ mol $l^{-1}$ when added to PMN (Atkinson et al 1977).

Table 5.3 Intra-Assay Variation

CR1 Sites/PMN

| | First Measurement | Second Measurement | Third Measurement | Mean±SD | % Variation from Mean | |
|---|---|---|---|---|---|---|
| Donor 1 | 34,159 | 39,879 | 37,863 | 37,300±2,901 | +7 | -8 |
| Donor 2 | 63,059 | 55,971 | 62,615 | 60,548±3,970 | +4 | -8 |

Note: The mean of the 3 measurements was calculated and the percentage variation of the highest and lowest measurement from the mean was calculated.

Colchicine

Colchicine is an alkaloid derived from the autumn crocus, Colchicium autumnal. It binds to tubulin dimers and causes depolymerisation of microtubules (MT). It was stored desiccated at 4°C. Prior to use a stock solution of $10^{-2}$ mol $l^{-1}$ in RPMI/BSA/EDTA was made and diluted to a final concentration of $10^{-5}$ mol $l^{-1}$ when added to PMN (Atkinson et al 1977).

Lummicolchicine

Lummicolchicine a functional analogue of colchicine, which does not affect MT structure, was stored desiccated at 4°C. Prior to use a stock solution of $10^{-2}$ $mol^{-1}$ in RPMI/BSA/EDTA was made and diluted to a final concentration of $10^{-5}$ mol $l^{-1}$ when added to PMN.

Cytochalasin B

Cytochalasin B derived from the fungus, Helminthosporium dematioideum is thought to bind to actin and dissociate microfilaments. It was stored protected from light at -70°C as a $10^{-2}$ mol $l^{-1}$ stock solution in DMSO. This stock solution was diluted to a final concentration of $10^{-5}$ mol $l^{-1}$ when added to PMN (Atkinson et al 1977).

Puromycin

Puromycin, a protein synthesis inhibitor, was stored desiccated at -20°C at 25mg/ml in deionised water. It was diluted to give a final concentration of 40$\mu$g/1x$10^{6}$ PMN (Rosso di San Secondo et al 1979).

Cycloheximide

Cycloheximide, a protein synthesis inhibitor, was stored desiccated at 4°C. Prior to use a stock solution of 5mg/ml was made in RPMI/BSA/EDTA. This was diluted to give a final concentration 200$\mu$l/1x$10^{6}$ (Rosso di San Secondo et al 1979).

DNP

DNP was stored desiccated at room temperature prior to use a stock solution of 3 mol $l^{-1}$ was made in RPMI/BSA/EDTA and diluted to a final concentration 3x$10^{-5}$ mol $l^{-1}$ when added to PMN.

Rotenone

Rotenone, which is light sensitive, was stored (protected from light) desiccated at -20°C. Prior to use a stock solution of $10^{-2}$ mol $l^{-1}$ was made in acetone and diluted to a final concentration of $10^{-4}$ mol $l^{-1}$ when added to PMN.

Antimycin A

Antimycin A was stored desiccated at -20°C. Prior to use a stock solution of 0.1%(w/v) was made in DMSO and diluted to a final concentration of 0.01%(w/v) when added to PMN.

DMSO

At the concentration used (0.1%(v/v)) DMSO had no effect on CR1 number (Table 5.4).

Trypan Blue

0.75g of trypan blue were dissolved in 100mls of normal saline to result in a 0.75(w/v) solution.

Increase in CR1 Number in Response to Warming to 37°C or the Addition of FMLP

Procedure

CR1 numbers on PMN are reported to increase spontaneously by warming to 37°C or by treatment with FMLP (Fearon and Collins 1983; Berger et al 1984; Richerson et al 1985).

PMN ($5x10^6$/ml) in RPMI/BSA/EDTA were incubated on ice for 30 min after which an aliquot was removed, centrifuged at 1,000g for 3min at 4°C, resuspended in RPMI/BSA/EDTA/I ($5x10^6$/ml) and CR1 numbers determined. The remaining cells were then incubated at 37°C in a shaking water bath for 30 min alone or in the presence of FMLP after which the cells were collected by centrifugation at 1,000g for 3 min at 4°C and resuspended in RPMI/BSA/EDTA/I ($5x10^6$/ml) and CR1 numbers determined.

Treatment of PMN with Drugs

PMN were treated with drugs to determine what effect protein synthesis inhibitors, energy inhibitors and inhibitors of cytoskeleton assembly would have on expression of CR1.

Procedure

PMN ($5x10^6$/ml) in RPMI/BSA/EDTA were incubated on ice for 30 min after which an aliquot was removed and CR1 numbers determined. The remaining cells were divided into separate aliquots and the following drugs were added, colchicine, lummicolchicine, cytochalasin B, puromycin, cycloheximide, DNP, rotenone and antimycin A. The incubation on ice was continued for 10 min to allow absorption of these drugs before the cells were transferred to a 37°C shaking water bath and incubated for 30 min. The cells were then centrifuged at 1,000g for 3min at 4°C, resuspended in RPMI/BSA/EDTA/I at $5x10^6$/ml and CR1 numbers determined.

Table 5.4 Effect of DMSO on PMN CR1 Numbers

| | CR1 Sites/PMN 37°C | 37°C + DMSO (0.1% (v/v)) | Percentage Change |
|---|---|---|---|
| Donor1 | 60,586 | 56,731 | -6 |
| Donor2 | 31,977 | 36,187 | +13 |

Viability of the PMN

After treatment with drugs a small aliquot of the PMN suspension was removed and diluted 1:20 with 0.75%(w/v) trypan blue. The cells were then mixed for 2 min before being placed on a slide and counted under a light microscope. The number of cells which excluded trypan blue were divided by the total number of cells counted and this number was expressed as a percentage. The proportion of viable cells in each PMN preparation was always greater than 95% when assessed by trypan blue exclusion.

## 5.8 Treatment of PMN with Trypsin

### Reagents

#### AB Plasma

One unit (200-250mls) of AB plasma was thawed at 37°C and the plasma placed into a volumetric flask. The plasma was allowed to clot by the addition of 4 mls of 1 mol $l^{-1}$ calcium chloride and the mixture stirred overnight at 4°C. The serum was separated from the clot by filtration through a Buchner funnel. The serum was heat inactivated at 56°C for 2 hours to destroy endogenous complement activity and dialysed against PBS (100x the serum volume) overnight at 4°C before ultracentrifugation at 28,000g to remove lipid and particulate material. The serum was then filtered through a sterile Millipore filter and stored at -20°C.

#### Trypsin

Trypsin was stored desiccated at -20°C. Prior to use, trypsin was made up to a stock solution of 10mg/ml in RPMI/BSA/EDTA and diluted to 0.2mg/1 x $10^6$ cells for use.

#### Buffers

PBS

PBS was made as previously described in chapter 2.6(c).

RPMI/BSA/EDTA/AB

A volume of RPMI/BSA/EDTA was made up containing 5%(v/v) AB serum. The AB serum was added to prevent clumping of the PMN.

RPMI/BSA/EDTA/AB/SBTI

A volume of RPMI/BSA/EDTA/AB was made up containing 0.1mg/ml SBTI.

Trypsinisation of PMN

PMN were trypsinised to remove all surface CR1 so that regeneration of CR1 could be studied.

Procedure

PMN were suspended in RPMI/BSA/EDTA/AB at $4x10^6$/ml and prewarmed to 37°C in a shaking water bath. Prewarmed trypsin solution was added to the cells and incubation continued for 15 min after which a 2-fold molar excess of SBTI was added to inactivate the enzyme. The cells were incubated for 2 min, then centrifuged at 1,000g for 5 min at 2°C. The cell pellet was given a further 3 washes in ice-cold RPMI/BSA/EDTA and the cells resuspended in RPMI/BSA/EDTA at $5x10^6$/ml.

Regeneration of PMN CR1 following Trypsinisation

An aliquot of trypsinised cells was removed immediately following trypsinisation and CR1 antigenic sites measured by RIA. The remaining PMN were incubated at 37°C in RPMI/BSA/EDTA/SBTI for 1 hour in a shaking water bath to allow regeneration of PMN CR1. When the incubation was complete the cells were centrifuged at room temperature for 5 min at 1,000g, resuspended in RPMI/BSA/EDTA/I ($5x10^6$/ml) and CR1 number determined by RIA.

To determine if PMN CR1 were regenerated at 0°C the cells were incubated on ice for 15 min following trypsinisation. After the incubation was complete the cells were centrifuged at 4°C for 5 min at 1000g and resuspended in RPMI/BSA/EDTA/I ($5x10^6$)/ml. CR1 number was then determined by RIA.

Inhibition of PMN CR1 Regeneration

After trypsinisation PMN were incubated at 37°C in RPMI/BSA/EDTA/AB/SBTI ($5x10^6$/ml) and puromycin, cycloheximide, cytochalasin B, or colchicine, at the same concentrations used previously (5.7) were added to the PMN suspension to see if they could inhibit the reappearance of CR1. After a 1 hour incubation the cell pellet was collected by centrifuged at 1,000g for 5 min at 0°C and given 3 washes in RPMI/BSA/EDTA/ before being resuspended RPMI/BSA/EDTA/I ($5x10^6$/ml and CR1 number determined by RIA.

5.9 Rosette Assays

Reagents

EAIgMC43b

EAIgMC43b were prepared as described in chapter 2.7(b).

EAIgG

EAIgG prepared with rabbit IgG anti-sheep red blood cells were kindly donated by Mrs J Veitch.

Vaseline

Vaseline for sealing the coverslips was melted at 80°C on a hot plate.

Buffers and Solutions

3%(w/v) Gluteraldehyde

3mls of gluteraldehyde was added to 100mls of normal saline.

HEM/EDTA/$NaN_3$

Hepes Earles Medium (HEM) containing EDTA (0.02 mol $l^{-1}$) and 0.2%(w/v) sodium azide ($NaN_3$). These inhibitors were added to prevent PMN proteases from cleaving C3b to iC3b. If this were to happen then both CR3 and CR1 rosettes would be measured (Ross et al 1983).

$GVB^{2+}$

$GVB^{2+}$ was prepared as described in chapter 2.7(b)

Procedure

Rosette assays were performed as an additional means of studying removal and regeneration of PMN CR1. Rosettes with EAIgG showed the proportion of PMN which bound EA by Fcγ receptors and rosettes with EAIgMC3b showed the proportion of PMN binding EAIgMC3b through CR1.

Before trypsinisation, immediately after trypsinisation and 1 hour after trypsinisation, PMN were standardised to $4x10^6$/ml in HEM/EDTA/$NaN_3$. Equal volumes of the PMN suspension (250µl) and $EAIgG_\lambda$ or EAIgMC3b ($1x10^8$/ml in $GVB^{2+}$) were mixed in round bottom test tubes and centrifuged at 200g for 5 min at room temperature. The cells were resuspended in the original supernatant and 500µl of 3%(w/v) gluteraldehyde were added to fix the cells. After incubation for 20 min at room temperature, 2mls of deionised water were added to the test tubes and the contents mixed by inversion. The cells were centrifuged at 200g for 5 min at room temperature and the supernatant discarded. The cells were resuspended in 250µl of 0.75%(w/v) trypan blue and a drop of this suspension was placed onto 3 glass slides. Rosettes were examined under coverslips sealed with melted vaseline. At least 200 cells were counted per slide and the mean percentage of cells forming rosettes with 3 or more E was calculated.

## 5.10 Patients with SLE and Normal Controls

15 patients with SLE (1 male and 14 females) (Tan et al 1982) and 14 healthy normal controls (5 males and 9 females) were included in the

study. Immediately prior to venous blood sampling the SLE patients were examined for evidence of disease activity, which was defined as continuing clinical activity of involved organ systems. These features included polyarthritis, rash, continuing active urinary sediment with cells and casts alone or in addition to proteinuria when present. Seven of the patients were in active phase of disease and 8 were in inactive disease at the time of study.

Fifty millilitres of venous blood were collected from each individual, 40mls of this were mixed with a 100 units of sodium heparin in a universal container and the PMN isolated as previously described (5.3). The isolated PMN were suspended in RPMI/BSA/EDTA at $5x10^6$/ml and PMN CR1 number was determined at 0°C, 37°C and in the presence of FMLP as previously described (5.7). The remaining 10mls of blood were centrifuged at 1000g for 5 min at 4°C and the plasma and buffy coat removed. The red cell pellet was then washed 3 times in RPMI/BSA, the cells were standardised to $2.5x10^8$/ml and E CR1 number determined by RIA.

## 5.11 Serial Studies

The CR1 number on E and PMN (at 37°C) were measured on 3 patients during periods of disease activity and also during remission.

Results

5.12 Quantification and Distribution of PMN CR1 at 37°C

A method for quantifying CR1 on PMN was successfully adapted from the method used for quantifying E CR1. Using this method the average number of PMN CR1 sites per cell on 33 normal individuals at 37°C was calculated to be 198,000 ± 33,000 using the polyclonal anti-CR1 $F(ab')_2$ antiserum. The average number on 14 normal individuals at 37°C was 75,000 ± 7,000 as determined by the monoclonal antibody E11. When the number of receptor sites per cell among these normal individuals were depicted as frequency histograms (Fig 5.2(a) and Fig 5.2(b)) they were distributed in a logarithmic fashion.

5.13 Expression of Different Pools of PMN CR1

The number of PMN CR1 sites per cell was calculated on 14 normal individuals using $^{125}I$-$F(ab')_2$ anti-CR1, at 0°C, 37°C and at 37°C in the presence of FMLP. At 0°C PMN expressed 87,000 ± 7,000 CR1 antigenic sites per cell, at 37°C 125,000 ± 16,000 CR1 sites per cell and in the presence of FMLP ($10^{-8}$ mol $l^{-1}$) 206,500 ± 21,000 sites per cell (Fig 5.3). The difference in CR1 number at 0°C and at 37°C was significantly different ($P<0.05$). The increase in expression of CR1 seen in the presence of FMLP was significantly different from the spontaneous increase at 37°C ($P<0.05$).

5.14 Effect of Protein Synthesis Inhibitors on Expression of CR1

PMN were stimulated with FMLP in the presence of cycloheximide and puromycin and CR1 numbers calculated using $^{125}I$-E11. Neither of these 2 protein synthesis inhibitors were found to have any effect on the stimulation of CR1 expression with FMLP (Fig 5.4) ($P>0.05$ in both cases). As protein synthesis inhibitors did not reduce CR1 expression in the presence of FMLP no studies were performed on the effect of these drugs on CR1 expression at 37°C.

5.15 Effect of Inhibitors of Cytoskeleton Assembly on Stimulation of CR1 Expression

Cytochalasin B and colchicine did not affect the increase in CR1 expression which was seen upon warming PMN to 37°C (Fig 5.5(a)) ($P>0.05$ in both cases). However both of these drugs abrogated the increased expression of CR1 seen in the presence of FMLP (Fig 5.5(b). Lummicolchicine a functional analogue of colchicine which does not affect microtubule structure (Wilson and Friedkin 1967) had no effect on FMLP stimulated expression of CR1 (Fig 5.6) ($P>0.05$). These results indicate that microtubule and microfilament assembly are important for the mobilisation of CR1 in response to FMLP. The specificity of the colchicine response is indicated by the observation that lummicolchicine did not prevent the FMLP induced increase in CR1 numbers.

Figure 5.2(a) Distribution of PMN CR1 in 33 Normal Individuals Determined by Polyclonal Anti-CR1 $F(ab')_2$

Legend

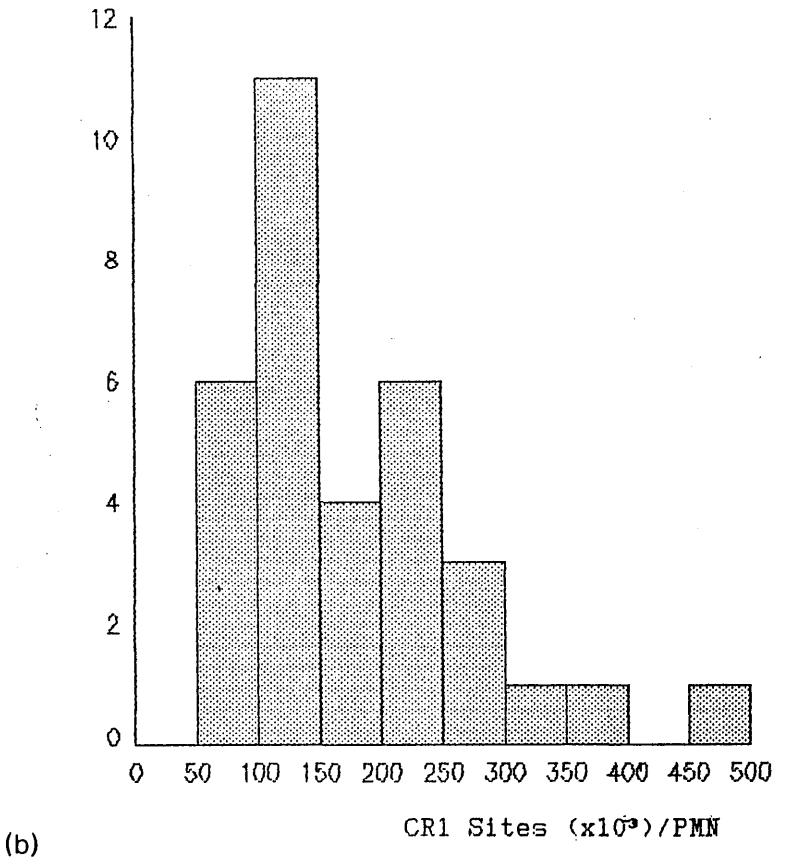
Histogram showing the frequency distribution of the number of binding sites on PMN for $^{125}I$-$F(ab')_2$ anti-CR1 among 33 normal individuals. The intervals are 50,000 sites/PMN.

Figure 5.2(b) Distribution of PMN CR1 in 14 Normal Individuals Determined by E11

Legend

Histogram showing the frequency distribution of the number of binding sites on PMN for $^{125}I$-E11 among 14 normal individuals. The intervals are 10,000 sites/PMN.

(a)


Number of Individuals
12
10
8
6
4
2
0
0 50 100 150 200 250 300 350 400 450 500
CR1 Sites (x10³)/PMN

(b)

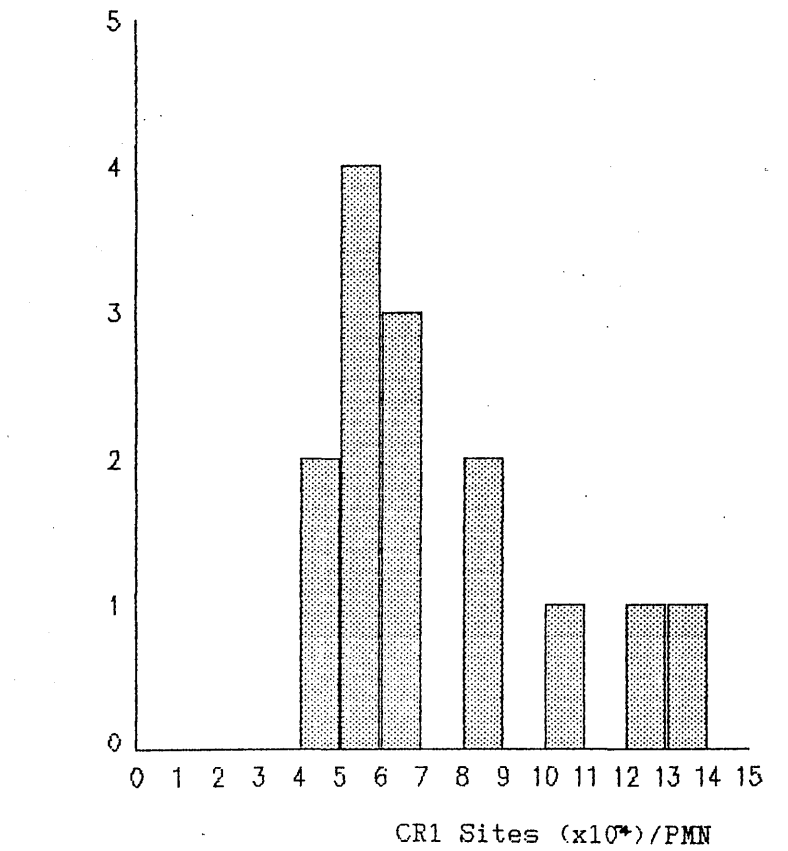

Number of Individuals
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
CR1 Sites (x10⁴)/PMN

Figure 5.3 Distribution of PMN CR1 at 0°C, 37°C and in the Presence of FMLP

Legend

Distribution of CR1 number (determined by polyclonal $F(ab')_2$ anti-CR1) on PMN from 14 normal individuals after incubation at 0°C, 37°C or in the presence of FMLP ($10^{-5}$ mol $l^{-1}$). The top of each bar represents the mean and the horizontal line represents the SEM.

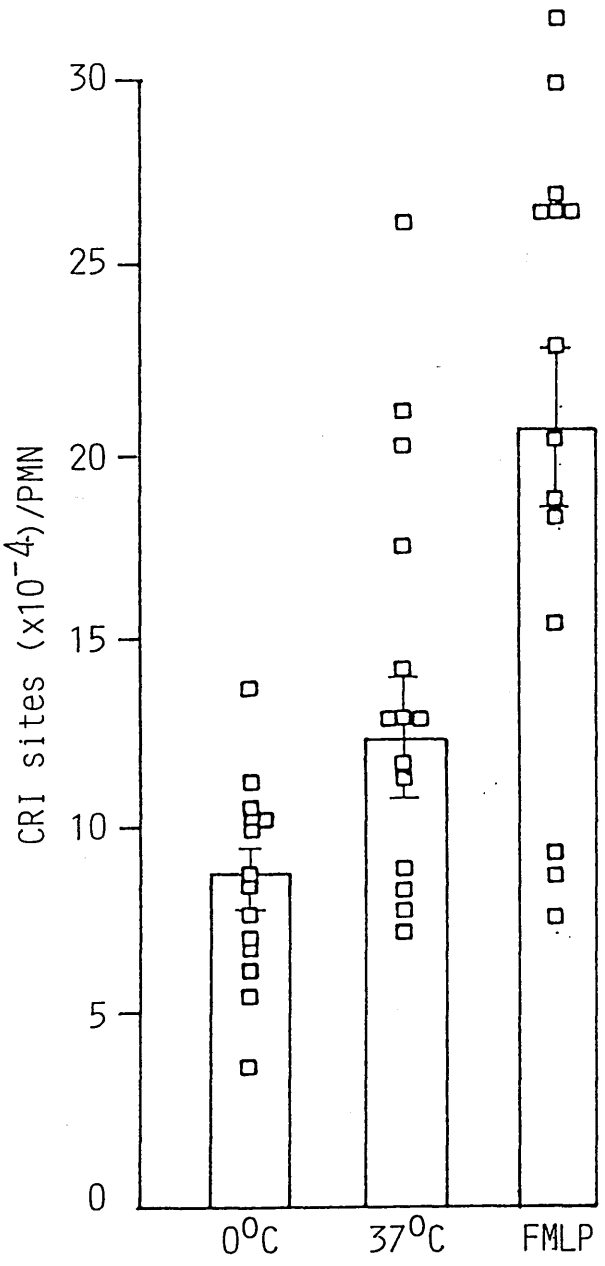

30
25
20
15
10
5
0
CRI sites (x10⁻⁴)/PMN
0°C
37°C
FMLP

Fig 5.4 Effect of Protein Synthesis Inhibitors on PMN CR1

Legend

Distribution of CR1 number (determined by E11) at 0°C, at 37°C, at 37°C in the presence of FMLP ($10^{-5}$ mol $l^{-1}$), and at 37°C in the presence of FMLP plus puromycin (40μg/1x$10^{6}$ cells), or cycloheximide 200μg/1x$10^{6}$ cells). Each bar represents the mean+SEM of 3 experiments.

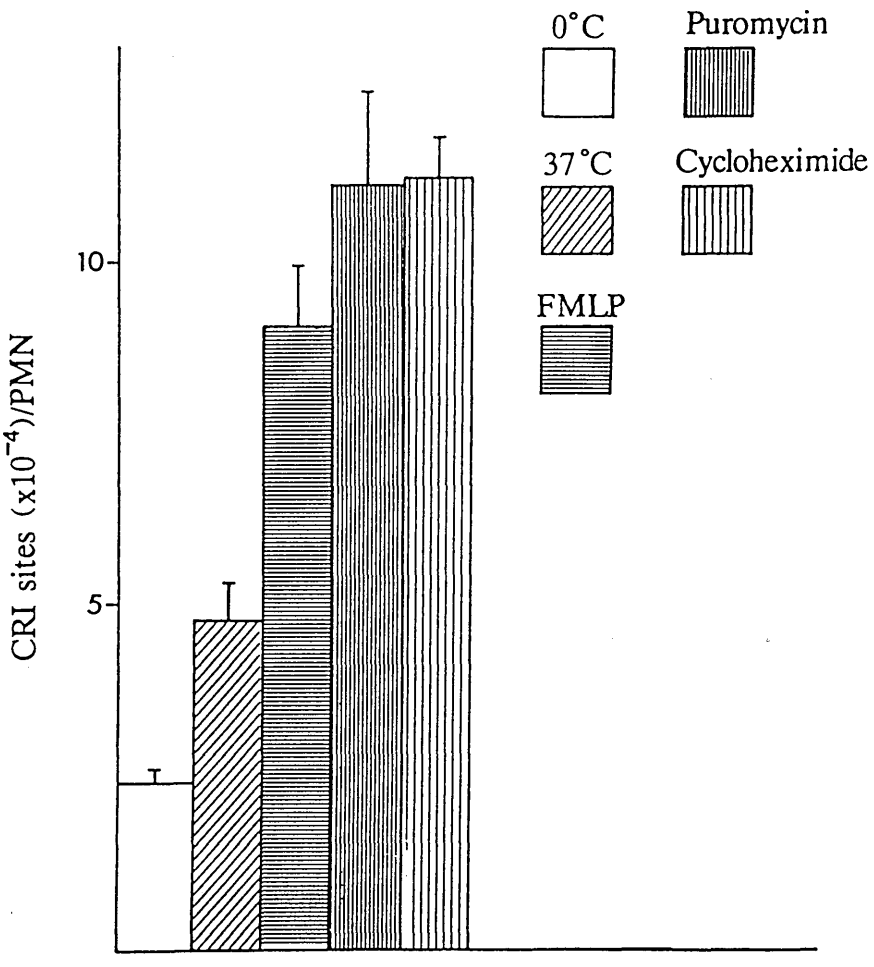

0°C
Puromycin
37°C
Cycloheximide
FMLP
10
5
CRI sites (x10$^{-4}$)/PMN

Fig 5.5(a) Effect of Inhibitors of Cytoskeleton Assembly on PMN CR1 at 37°C

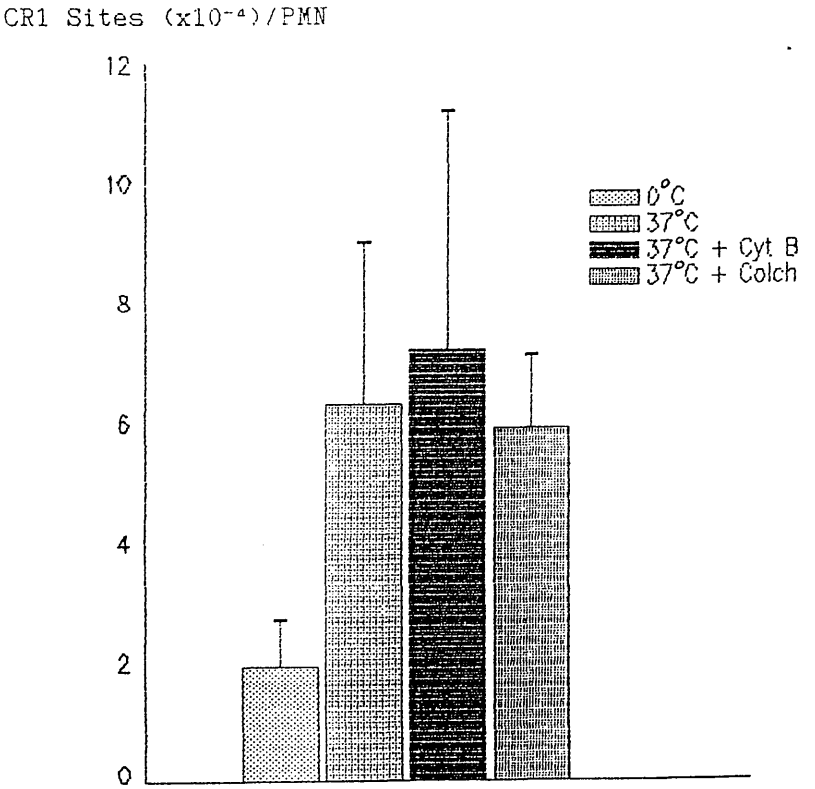
Legend

PMN CR1 number was measured using $^{125}$I-E11 at 0°C and at 37°C in the presence of colchicine ($10^{-5}$ mol $l^{-1}$) and cytochalasin B ($10^{-5}$ mol $l^{-1}$). The top of each bar represents the mean of 4 experiments and the vertical line the SEM.

Fig 5.5(b) Effect of Inhibitors of Cytoskeleton Assembly on PMN CR1 in the presence of FMLP

Legend

Distribution of CR1 number (determined by polyclonal $F(ab')_2$ anti-CR1) at 0°C, at 37°C, at 37°C in the presence of FMLP ($10^{-5}$ mol $l^{-1}$) and at 37°C in the presence of FMLP plus colchicine ($10^{-5}$ mol $l^{-1}$) or cytochalasin B (cyt B $10^{-5}$ mol $l^{-1}$). Each bar represents the mean ± SEM of 9 experiments.


CR1 Sites (x10⁻⁴)/PMN
12
10
8
6
4
2
0
0°C
37°C
37°C + Cyt B
37°C + Colch

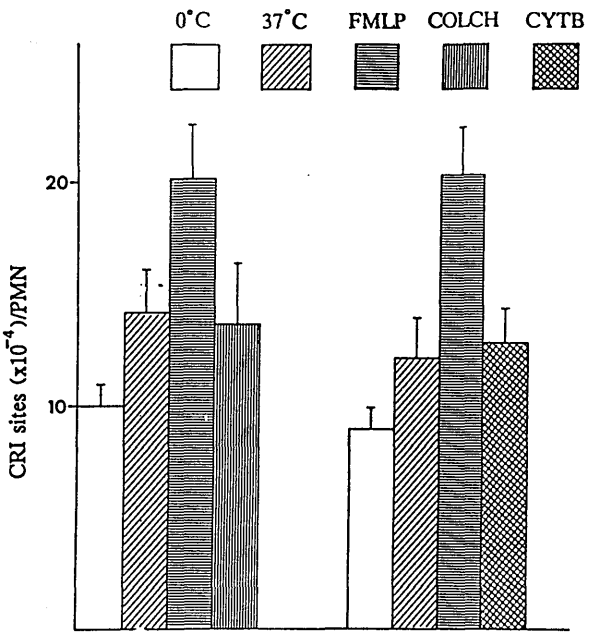
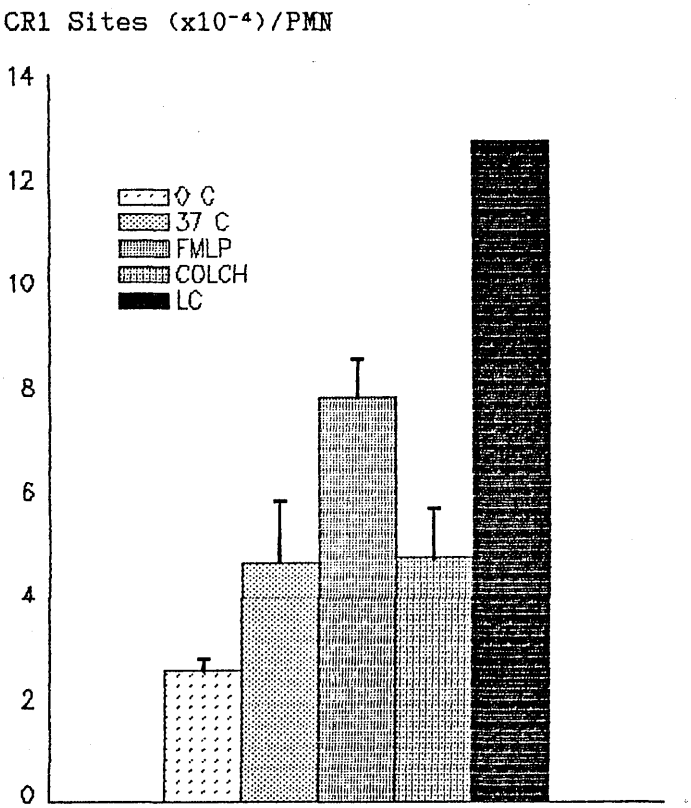

0°C
37°C
FMLP
COLCH
CYTB
CRI sites (x10⁻⁴)/PMN
20
10

Fig 5.6 Effect of Lummicolchicine on PMN CR1 Expression

Legend

PMN CR1 number was measured using $^{125}$I-E11 at 0°C), 37°C, 37°C in the presence of FMLP ($10^{-5}$ mol $l^{-1}$) and at 37°C in the presence of FMLP plus colchicine ($10^{-5}$ mol $l^{-1}$) or lummicolchicine ($10^{-5}$ mol $l^{-1}$). The top of each bar represents the mean of 3 experiments and the vertical line the SEM.


CR1 Sites (x10⁻⁴)/PMN
14
12
10
8
6
4
2
0
0 C
37 C
FMLP
COLCH
LC

## 5.16 Suppression of FMLP Induced Increase of CR1 with Energy Inhibitors

The energy inhibitors, DNP, rotenone and antimycin A did not affect the increased expression of PMN CR1 which was seen upon warming the cells from 0°C to 37°C (Fig 5.7(a)) ($P>0.05$ for all inhibitors). However in the presence of FMLP these drugs inhibited the FMLP induced expression of CR1 at 37°C (Fig 5.7(b)) ($P<0.05$ for all inhibitors).

## 5.17 Effect of Trypsin on PMN CR1

Incubation of PMN with 0.08%(w/v) trypsin removed all CR1 detectable by radioimmunoassay using $F(ab')_2$ anti-CR1 (Fig 5.8). After incubation at 37°C for 1 hour the surface expression of CR1 was restored almost to normal levels (Fig 5.8) ( $P<0.05$). CR1 number was not restored if PMN were incubated at 0°C (Table 5.5). That CR1 were being removed was substantiated by the finding that while the percentage of PMN rosetting with EAIgG (which measures the binding of the PMN Fcγ receptors) remained the same before and after trypsinisation, the percentage of PMN rosetting with EAIgMC3b (which measures the binding of the PMN CR1) fell significantly (Table 5.6). After incubation for 1 hour at 37°C the percentage of PMN rosetting with EAIgMC3b increased significantly, again indicating that CR1 were being translocated from an intracellular site to the cell surface (Table 5.6).

## 5.18 Effect on Drugs on the Regeneration of CR1 on Trypsinised PMN

Addition of the protein synthesis inhibitors puromycin or cycloheximide to trypsinised PMN did not affect the reexpression of CR1 which was seen after 1 hour (Fig 5.9) ($P>0.05$ in both cases). Addition of colchicine or cytochalasin B to trypsinised PMN also had no effect on the return of CR1 to the membrane over 1 hour (Fig 5.10) ($P>0.05$ in both cases)

## 5.19 CR1 Numbers on PMN and E from Patients with SLE

The mean number of E CR1 on 9 normal individuals was 2,516 ± 826 sites per cell whereas the mean number of E CR1 from the 7 patients with active disease was significantly lower (393 ± 370 sites per cell) ($P<0.001$). This number was also significantly lower than the mean number of E CR1 from the 8 patients with inactive disease (2,795 ± 814 sites/cell) $P<0.001$ (Fig 5.11). The difference in E CR1 numbers between normal individuals and those with inactive SLE was not significant ($P>0.05$). This reduction of E CR1 in SLE patients with active disease is in agreement with the findings of others (Ross et al 1985; Holme et al 1986).

The number of CR1 sites on patients' PMN was measured at 0°C and at 37°C in the absence and presence of FMLP. In patients with inactive SLE the numbers of CR1 sites at those temperatures were 124,528 ± 37,298, 165,672 ± 62,932 and 209,344 ± 57092 respectively (Fig 5.12). These

5.7(a) Effect of Energy Inhibitors on PMN CR1 Expression at 37°C

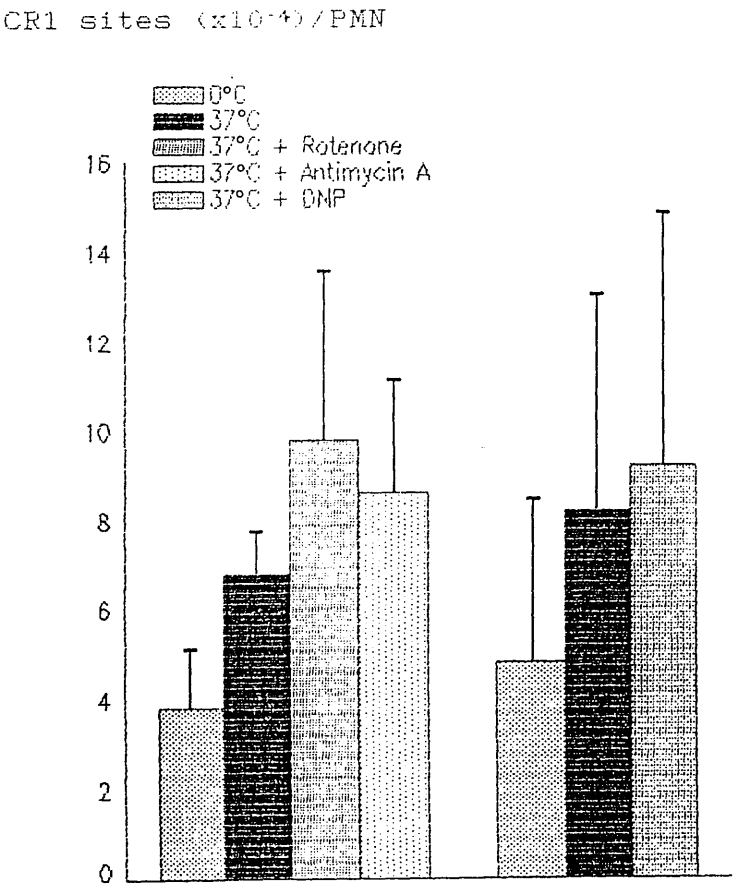
Legend

PMN CR1 expression was measured using $^{125}$I-E11 at 0°C, 37°C and at 37°C in the presence of rotenone ($10^{-4}$ mol $l^{-1}$), antimycin A (0.01%) or DNP ($3x10^{-5}$ mol $l^{-1}$). The top of each bar represents the mean of 3 experiments and the vertical line represents the SEM.

5.7(b) Effect of Energy Inhibitors on PMN CR1 expression in the Presence of FMLP

Legend

Distribution of CR1 number at 0°C, at 37°C, at 37°C in the presence of FMLP ($10^{-5}$ mol $l^{-1}$) and at 37°C in the presence of FMLP plus DNP ($3x10^{-5}$ mol $l^{-1}$) or rotenone ($10^{-4}$ mol $l^{-1}$) or antimycin A (0.01%(w/v)). CR1 number for rotenone and antimycin A treated PMN were determined by E11 and CR1 number for DNP treated PMN was determined by polyclonal $F(ab')_2$ anti-CR1. Each bar represents the mean±SEM of 5 experiments.


CR1 sites (x10⁻⁴)/PMN
0°C
37°C
37°C + Rotenone
37°C + Antimycin A
37°C + DNP
16
14
12
10
8
6
4
2
0

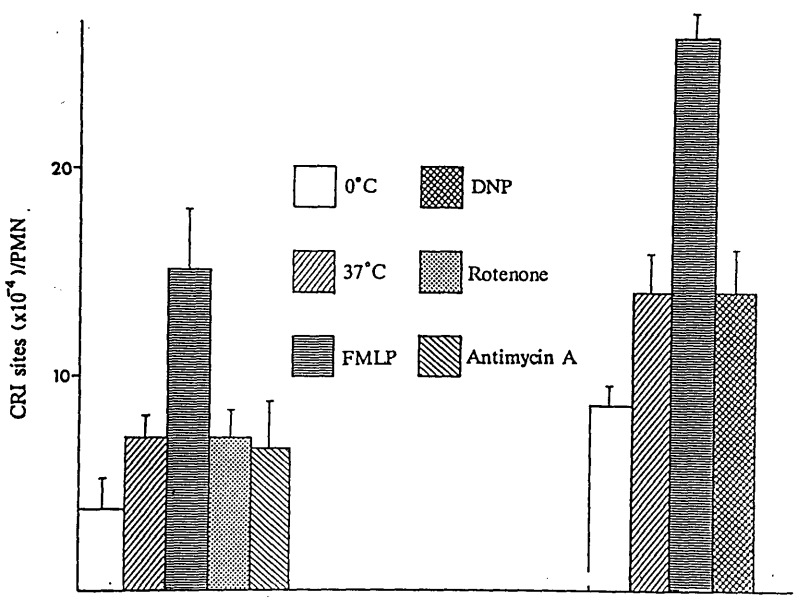

CRI sites (x10⁻⁴)/PMN
20
10
0°C
37°C
FMLP
DNP
Rotenone
Antimycin A

Fig 5.8 Incubation of Trypsinised PMN for 1 Hour at 37°C

Legend

PMN CR1 number was measured on control cells at 37°C, on trypsinised PMN immediately after trypsinisation and after the trypsinised cells had been incubated at 37°C for 1 hour. The top of each bar represents the mean of 12 experiments and the vertical lines the SEM. CR1 was measured using $^{125}$I-F(ab')$_2$ anti-CR1.

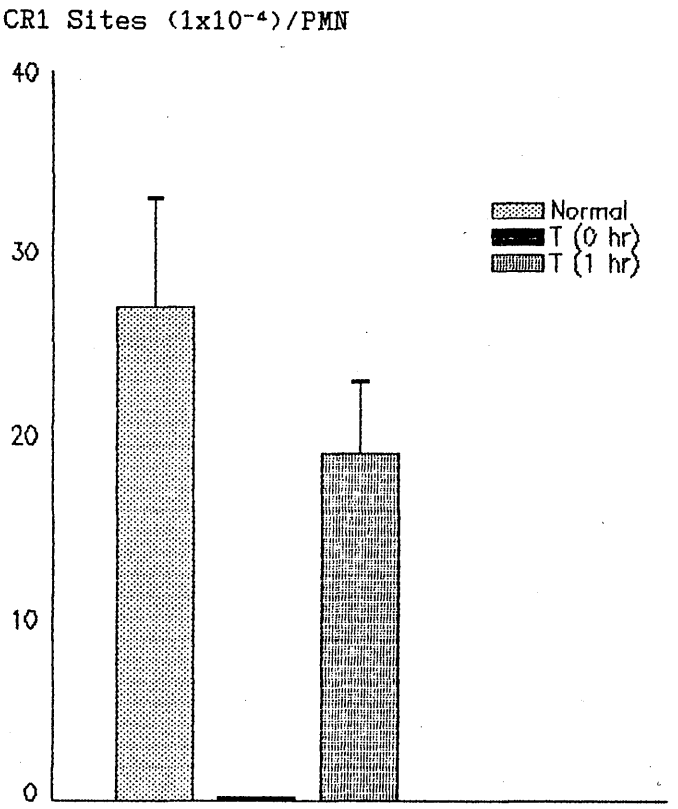

CR1 Sites (1x10⁻⁴)/PMN
40
30
20
10
0
Normal
T (0 hr)
T (1 hr)

Table 5.5 CR1 Antigenic Sites on PMN

| | CR1 Antigenic Sites on PMN | | | |
|---|---|---|---|---|
| | Normal(37°C) | T⁰ | 15 min(37°C) | 15 min(0°C) |
| Donor 1 | 232,434 | 20,313 | 97,544 | 20,299 |
| Donor 2 | 94,196 | 0 | 36,960 | 0 |
| Donor 3 | 820,403 | 0 | 202,908 | 0 |

Note: T⁰ = Trypsinised PMN at time 0 hours.

Table 5.6 PMN Rosettes with EAIgMC3b and EAIgG

| | Percentage of PMN Rosetting with: | | | | | |
|---|---|---|---|---|---|---|
| | EAIgMC3b | TEAIgMC3b | TEAIgMC3b (1hr incub) | EAIgG | TEAIgG | TEAIgG (1hr incub) |
| Donor 1 | 27±8 | 9±1 | 27±4 | 66±12 | 75±10 | 70±10 |
| Donor 2 | 32±5 | 4±2 | 24±4 | 65±5 | 61±6 | 66±4 |
| Donor 3 | 49±5 | 5±1 | 11±1 | 73±5 | 73±8 | 80±8 |

Note: T = Trypsinised PMN; 1hr incub = 1 hour incubation

## 5.9 Effect of Protein Synthesis Inhibitors on Trypsinised PMN

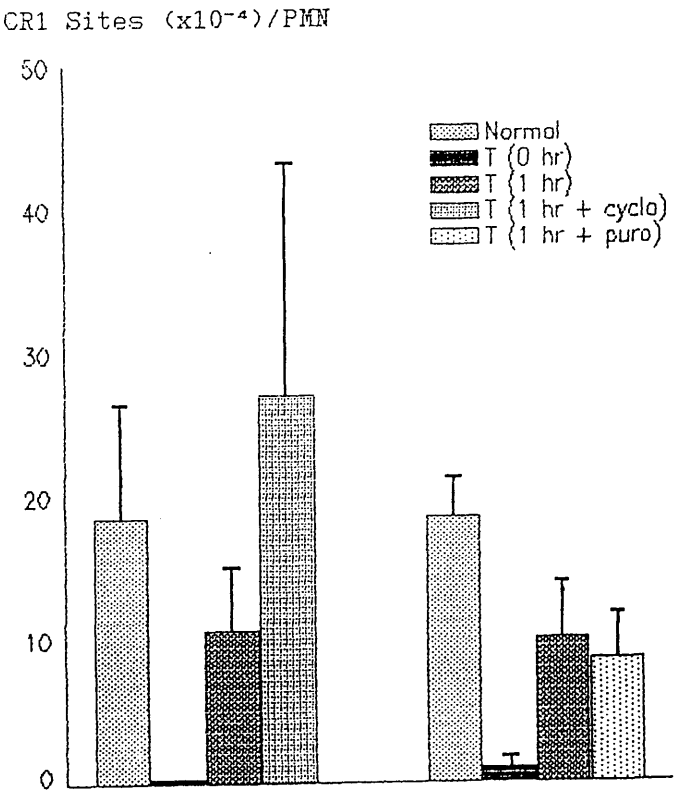
Legend

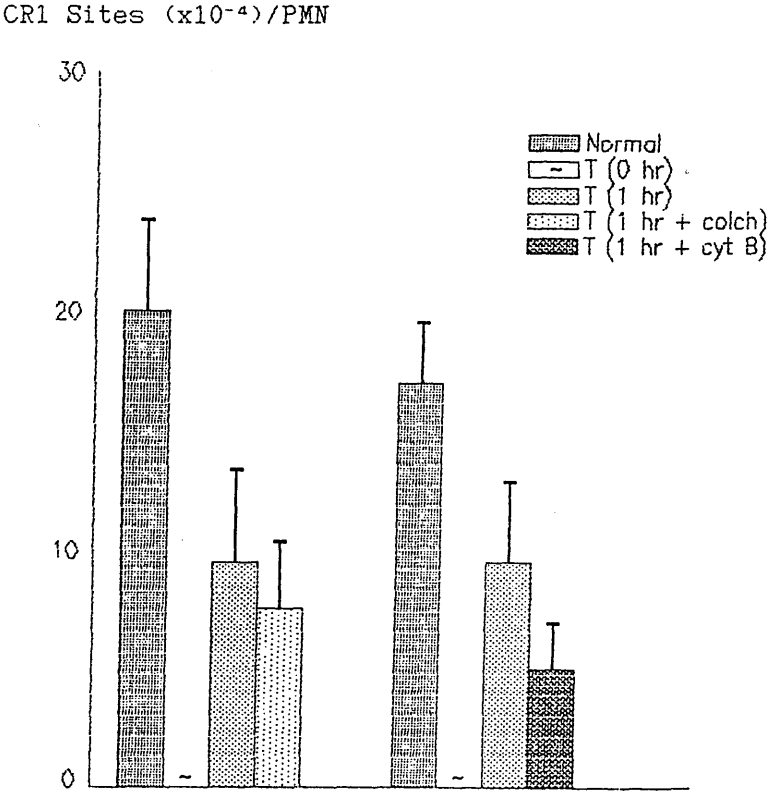
PMN CR1 was measured on control cells at 37°C, on trypsinised PMN immediately after trypsinisation and on trypsinised PMN after a 1 hour incubation at 37°C alone or in the presence of cycloheximide (200μg/1x$10^6$ cells) or puromycin (40μg/1x$10^6$ cells). CR1 was measured using $^{125}$I-F(ab')$_2$ anti-CR1. The top of each bar represents the mean of 4 experiments and the vertical lines the SEM.

## 5.10 Effect of Inhibitors of Cytoskeleton Assembly on Trypsinised PMN

Legend

PMN CR1 number was measured on control cells at 37°C, on trypsinised PMN immediately after trypsinisation and on trypsinised PMN after a 1 hour incubation alone or in the presence of colchicine ($10^{-5}$ mol $l^{-1}$) or cytochalasin B ($10^{-5}$ mol $l^{-1}$). The top of each bar represents the mean of 4 experiments and the vertical lines the SEM. CR1 was measured using $^{125}$I-F(ab')$_2$ anti-CR1.


CR1 Sites (x10⁻⁴)/PMN
50
40
30
20
10
0
Normal
T (0 hr)
T (1 hr)
T (1 hr + cyclo)
T (1 hr + puro)


CR1 Sites (x10⁻⁴)/PMN
30
20
10
0
Normal
T (0 hr)
T (1 hr)
T (1 hr + colch)
T (1 hr + cyt B)

5.11 E CR1 on SLE Patients

Legend

Distribution of CR1 on E determined by polyclonal $F(ab')_2$ anti-CR1 on 9 normal individuals, on 7 SLE patients with active disease and on 8 SLE patients with inactive disease.

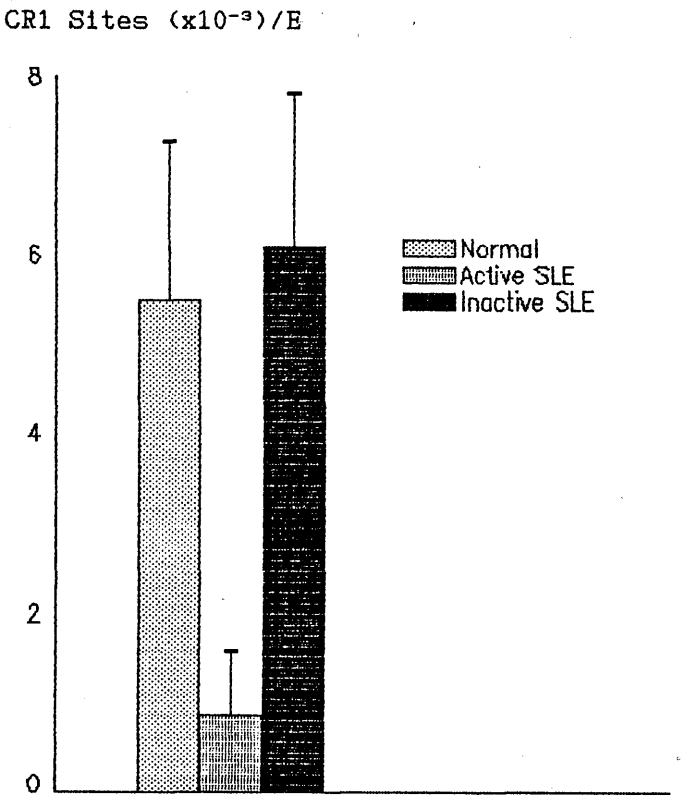
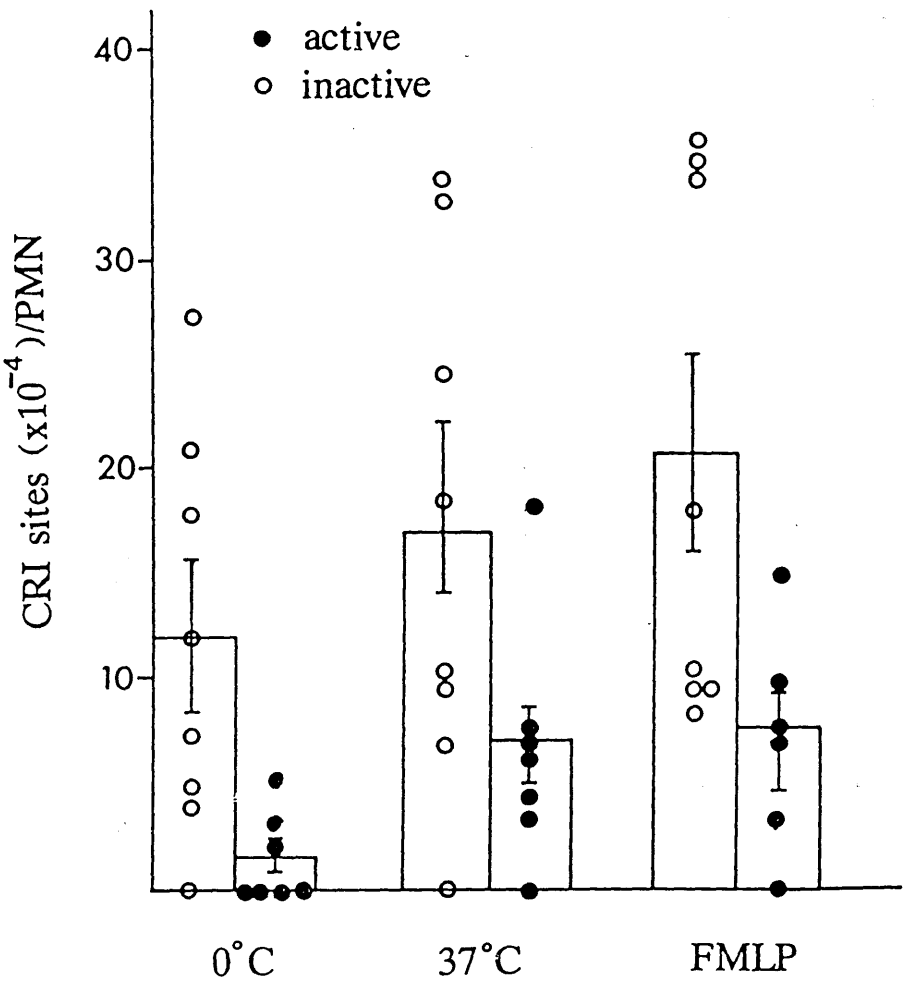

CR1 Sites (x10⁻³)/E
8
6
4
2
0
Normal
Active SLE
Inactive SLE

Fig 5.12 PMN CR1 Number on SLE Patients

Legend

Distribution of CR1 number determined by polyclonal $F(ab')_2$ anti-CR1 on PMN from 7 SLE patients with active disease (●), and 8 with inactive disease (○) after incubation at 0°C, 37°C or in the presence of FMLP ($10^{-5}$ mol $l^{-1}$). Assays on FMLP treated PMN were performed on only 6 patients with active SLE.


active
inactive
40
30
20
10
CRI sites (x10$^{-4}$)/PMN
0°C
37°C
FMLP

numbers were not significantly lower than the numbers expressed on normal individuals (Fig 5.3) ($P > 0.05$ in all cases).

However in patients with active SLE the numbers expressed at 0°C and at 37°C in the absence and presence of FMLP were 15,394 ± 7809, 68,388 ± 2,2921 and 69,361 ± 2,2972 respectively (Fig 5.12). These numbers were significantly lower than normal PMN CR1 and lower than PMN CR1 patients with inactive SLE ($P<0.05$ in all cases). In the SLE patients with active disease the level of expression of CR1 following exposure to FMLP was not significantly different from that expressed at 37°C.

## 5.20 Serial Studies

CR1 numbers on E and PMN were measured on 3 patients during periods of disease activity and also during remission. In the active state CR1 numbers were low on both E and PMN whereas they increased on both cell types when the disease became inactive (Table 5.7).

## 5.21 Correlation Between CR1 Sites on E and PMN

In normal individuals there was a significant correlation ($r = 0.77$) between the number of CR1 expression on PMN at 37°C and the number of CR1 sites per E ($P < 0.05$), (Fig 5.13(a)). Similar results were found in SLE patients where the number of PMN CR1 expressed at 0°C, at 37°C and in the presence of FMLP correlated significantly with the number of CR1 sites per E ($r = 0.57$, 0.57 and 0.59 respectively), (Fig 5.13(b),(c) and(d)), ($P < 0.05$ in all cases). In SLE patients correlations were found between PMN CR1 expression at 0°C and at 37°C ($r = 0.945$; $P < 0.001$), (Fig 5.14(a)), between CR1 expression at 0°C and in the presence of FMLP ($r = 0.954$; $P < 0.001$), (Fig 5.14(b)) and between CR1 expression at 37°C and in the presence of FMLP ($r = 0.96$; $P < 0.001$), (Fig 5.14(c)).

In normals correlations were not significant between PMN CR1 expression at 0°C and at 37°C ($r = 0.434$; $P > 0.05$), (Fig 5.15(a)) or between PMN CR1 expression at 0°C and in the presence of FMLP ($r = 0.434$; $P > 0.05$), (Fig 5.15(b)). However there was a significant correlation between expression at 37°C and in the presence of FMLP ( $r=0.7055$; $P < 0.01$), (Fig 5.15(c)).

Table 5.7 Serial Study

| | CR1 sites/cell | | | |
|---|---|---|---|---|
| | PMN | | E | |
| Patient | Active | Inactive | Active | Inactive |
| 1 | 41,847 | 53,146 | 0 | 440 |
| 2 | 0 | 257,927 | 361 | 2859 |
| 3 | 70,048 | 185,669 | 2876 | 9526 |

Fig 5.13(a) Correlation between PMN CR1 Expression at 37°C and E CR1 Expression in Normals

Legend

The number of CR1 sites on the E and PMN of 9 normal individuals was determined and then the number of CR1 sites per E was plotted against the number of PMN CR1 sites. The line obtained from linear regression analysis had a significant correlation coefficient. ($r = 0.77$; $P < 0.05$).

Fig 5.13(b) Correlation between PMN CR1 Expression at 0°C an E CR1 Expression in SLE Patients

Legend

The number of CR1 sites on the E and PMN of 13 patients with SLE was determined and then the number of CR1 sites per E was plotted against the number of PMN CR1 sites. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.57$; $P < 0.05$).

Fig 5.13(c) Correlation between PMN CR1 Expression at 37°C and E CR1 Expression in SLE Patients

Legend

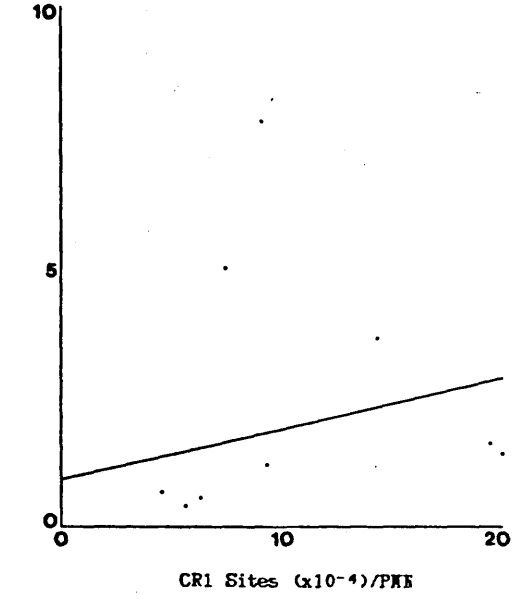
The number of CR1 sites on the E and PMN of 8 patients with SLE was determined and then the number of CR1 sites per E was plotted against the number of PMN CR1 sites. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.57$; $P < 0.05$).

Fig 5.13(d) Correlation between PMN CR1 Expression in the Presence of FMLP and E CR1 Expression in SLE Patients

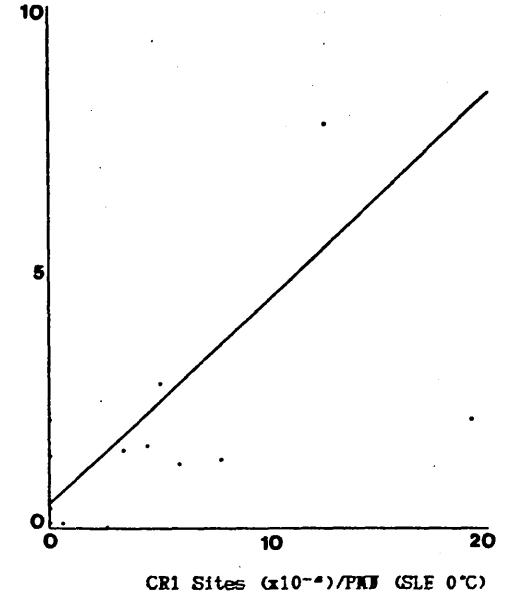
Legend

The number of CR1 sites on the E and PMN of 10 patients with SLE was determined and then the number of CR1 sites per E was plotted against the number of PMN CR1 sites. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.59$; $P < 0.05$).

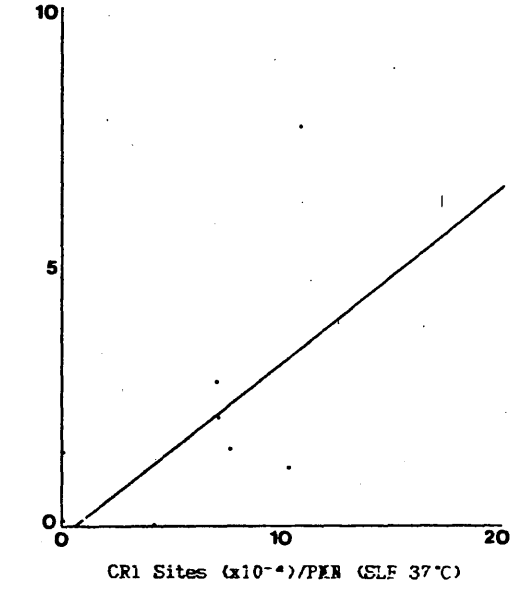
(a)


CR1 Sites (x10⁻³)/E
10
5
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN

(b)


CR1 Sites (x10⁻³)/E (SLE)
10
5
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (SLE 0°C)

(c)


CR1 Sites (x10⁻³)/E (SLE)
10
5
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (SLE 37°C)

(d)

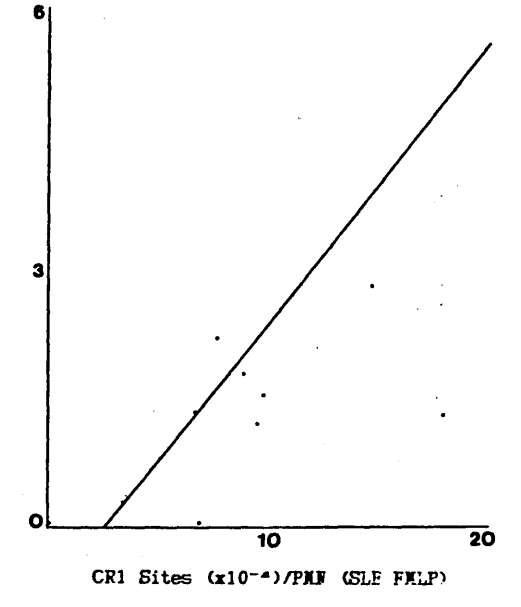

CR1 Sites (x10⁻³)/E (SLE)
6
3
0
10
20
CR1 Sites (x10⁻⁴)/PMN (SLE FMLP)

Fig 5.14(a) Correlation between PMN CR1 Expression at 0°C and at 37°C in SLE Patients

Legend

The number of CR1 sites on the PMN of 8 patients with SLE was determined at 0°C and at 37°C. The number of PMN CR1 sites at 37°C was then plotted against the number of PMN CR1 sites at 0°C. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.945$; $P < 0.001$).

Fig 5.14(b) Correlation between PMN CR1 Expression at 0°C and in the Presence of FMLP in SLE Patients

Legend

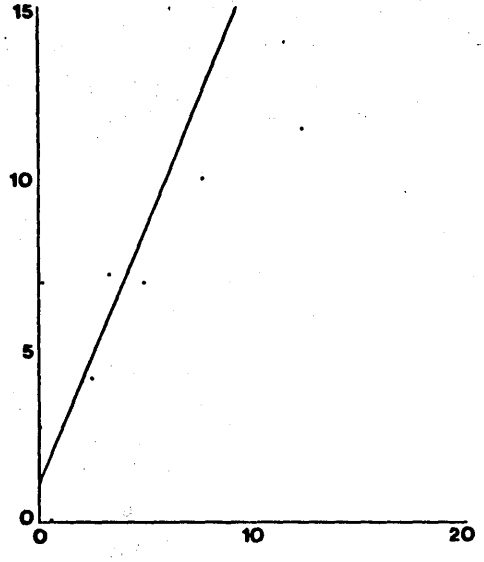
The number of CR1 sites on the PMN of 10 patients with SLE was determined at 0°C and in the presence of FMLP. The number of PMN CR1 sites calculated in the presence of FMLP was then plotted against the number of PMN CR1 sites at 0°C. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.954$; $P < 0.001$).

Fig 5.14(c) Correlation between PMN CR1 Expression at 37°C and in the Presence of FMLP in SLE Patients

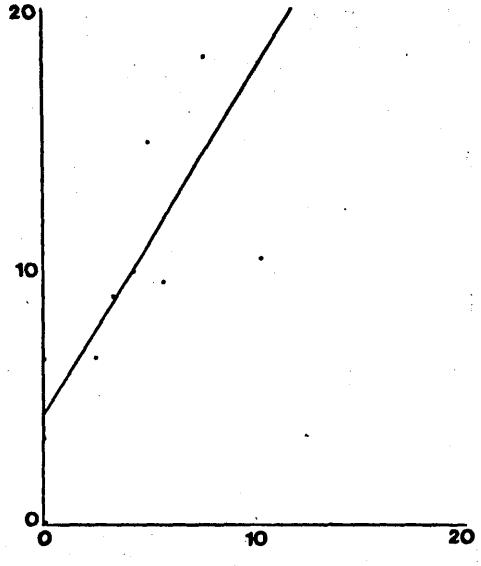
Legend

The number of CR1 sites on the PMN of 9 patients with SLE was determined at 37°C and in the presence of FMLP. The number of PMN CR1 sites calculated in the presence of FMLP was then plotted against the number of PMN CR1 sites at 37°C. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.96$; $P < 0.001$).

(a)


CR1 Sites (x10⁻⁴)/PMN (SLE 37°C)
15
10
5
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (SLE 0°C)

(b)


CR1 Sites (x10⁻⁴)/PMN (SLE FMLP)
20
10
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (SLE 0°C)

(c)

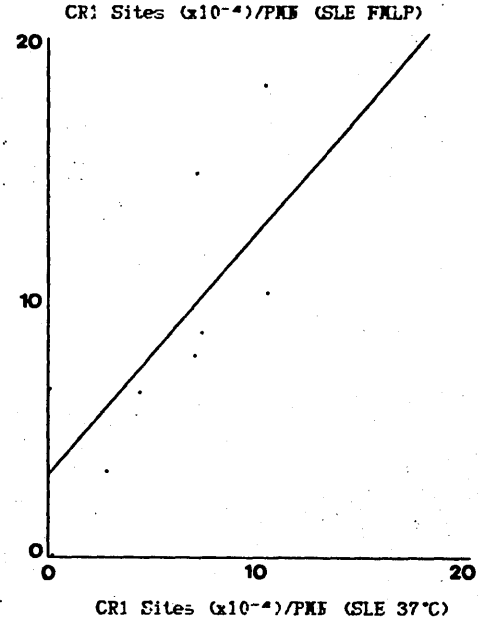

CR1 Sites (x10⁻⁴)/PMN (SLE FMLP)
20
10
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (SLE 37°C)

Fig 5.15(a) Correlation between PMN CR1 Expression at 0°C and at 37°C in Normals

Legend

The number of CR1 sites on the PMN of 12 normal individuals was determined at 0°C and at 37°C. The number of PMN CR1 sites at 37°C was then plotted against the number of PMN CR1 sites at 0°C. The line obtained from linear regression analysis did not have a significant correlation coefficient ($r = 0.434$; $P > 0.05$).

Fig 5.15(b) Correlation between PMN CR1 Expression at 0°C and in the Presence of FMLP in Normals

Legend

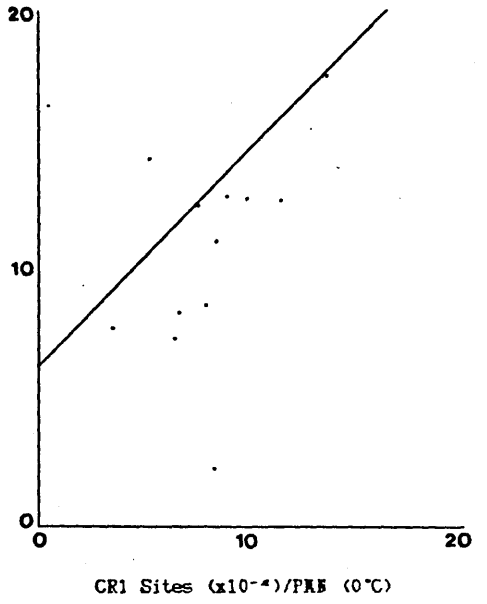
The number of CR1 sites on the PMN of 12 normal individuals was determined at 0°C and in the presence of FMLP. The number of PMN CR1 sites calculated in the presence of FMLP was then plotted against the number of PMN CR1 sites at 0°C. The line obtained from linear regression analysis did not have a significant correlation coefficient ($r = 0.434$; $P > 0.05$).

Fig 5.15(c) Correlation between PMN CR1 Expression at 37°C and in the Presence of FMLP in Normals

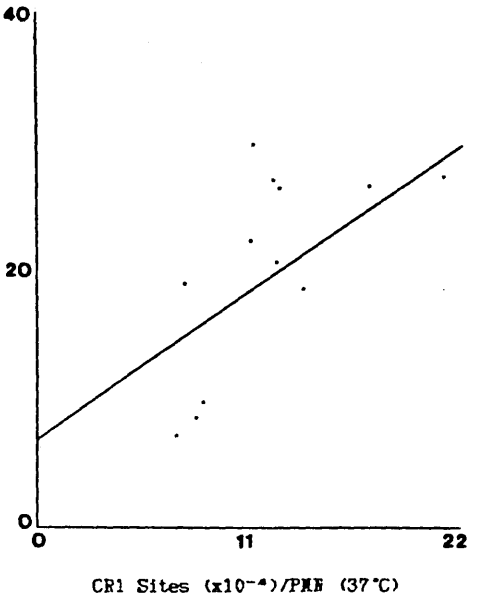
Legend

The number of CR1 sites on the PMN of 14 normal individuals was determined at 37°C and in the presence of FMLP. The number of PMN CR1 sites calculated in the presence of FMLP was then plotted against the number of PMN CR1 sites at 37°C. The line obtained from linear regression analysis had a significant correlation coefficient ($r = 0.7055$; $P < 0.01$).

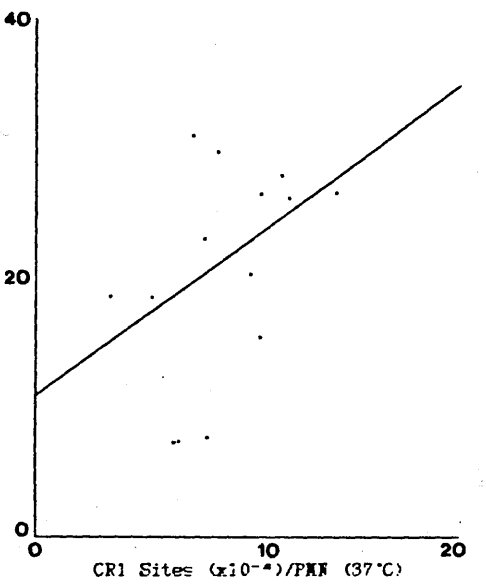
(a)


CR1 Sites (x10⁻⁴)/PMN (37°C)
20
10
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (0°C)

(b)


CR1 Sites (x10⁻⁴)/PMN (FMLP)
40
20
0
0
11
22
CR1 Sites (x10⁻⁴)/PMN (37°C)

(c)


CR1 Sites (x10⁻⁴)/PMN (FMLP)
40
20
0
0
10
20
CR1 Sites (x10⁻⁴)/PMN (37°C)

## 5.22 Discussion

The average number of CR1 sites per PMN on 33 normal individuals calculated at 37°C using a polyclonal antiserum was 197,715 ± 33,577 (range 50,000-500,000). This is higher than that reported by Fearon who using a polyclonal antiserum found only 57,000 CR1 sites per PMN. However this was calculated on 1 sample and falls within the range of PMN numbers quoted above. Using a monoclonal anti-CR1 antibody, 57F, Iida et al (1982) reported that there were 140,000 CR1 sites per PMN. This calculation was performed on PMN which had been detergent solubilised therefore intracellular CR1 would have been included in this estimation. Hogg et al (1984) reported an average of 46,000 CR1 sites per PMN on 4 normal individuals at room temperature. I have found using the same monoclonal, that on 14 normal individuals the average number of CR1 sites per PMN was 75,558 ± 7,613 at 37°C (range 40,000-140,000). The higher temperature which I calculated PMN CR1 may have been responsible for this difference.

The range of CR1 sites per PMN calculated by both methods was large. A large range of CR1 sites is also found on E. (Wilson et al 1982; Holme et al 1986).

The distribution of PMN CR1 number calculated by both polyclonal and monoclonal antisera was logarithmic (Fig 5.2(a)&(b)) like that found on E by Holme et al (1986). Studies on the distribution of E CR1 have yielded conflicting results. In some, E CR1 levels were reported to be trimodally distributed (Wilson et al 1982; Minota et al 1984; Nojima et al 1985). As previously described in chapter 3.1 this distribution originally led to the hypothesis that CR1 levels on E were inherited with the level of expression being controlled by 2 codominant alleles at a single locus. More recent studies have found that E CR1 are distributed in a normal fashion (Walport et al 1985; Ross et al 1985).

It would be interesting to determine which of these categories of distribution PMN CR1 falls into. Although the size of sample in my own study was small in comparision to the size of samples used in E studies, as the number of E CR1 and PMN CR1 number on normals appears to correlate (r=0.77) the distribution of PMN CR1 probably parallels E CR1 distribution in being distributed in a logarithmic fashion (Holme et al 1986).

In agreement with the work of others (Fearon & Collins 1983; Berger et al 1984; Richerson et al 1985) I have found that PMN CR1 expression can be increased by warming PMN to 37°C and further increased by stimulation with FMLP ($10^{-8}$ Mol $l^{-1}$) (Fig 5.3). This increased expression was not as a result of *de novo* protein synthesis as it was not inhibited by the presence of puromycin or cycloheximide indicating that the additional receptors were probably being translocated from an intracellular pool such as was described by O'Shea et al (1985a).

The results with the energy inhibitors DNP, rotenone and antimycin A and inhibitors of cytoskeleton assembly, colchicine and cytochalasin B suggest that CR1 which are brought to the surface upon warming to 37°C come from a different source than do CR1 which are expressed after stimulation with FMLP. DNP is a drug which uncouples the election transport chain from the production of energy by preventing the phosphorylation of ADP to ATP. Rotenone and antimycin A both inhibit energy production by blocking electron transport flow between the reduced form of nicotinamide adenine dinucleotide (NADH) and cytochrome b and between cytochrome b and cytochrome c respectively. In the presence of these drugs expression of CR1 at 37°C was normal (Fig 5.7(a)). However, the increased expression following exposure to FMLP did not occur (Fig 5.7(b)). This finding implies that translocation of CR1 from an internal pool is an energy dependent process, while CR1 brought to the surface in response to warming to 37°C does not require energy.

In support of the theory that intracellular CR1 comes from 2 different sources it was found that cytochalasin B and colchicine had no effect on the expression of CR1 at 37°C while they abrogated the increase seen in the presence of FMLP at 37°C. Therefore an intact cytoskeleton was not required for the spontaneous increase (Fig 5.5(a)) at 37°C but it was required for the mobilisation of CR1 in response to FMLP.

The cytoskeleton has been shown to play a role in the distribution of CR1 in the plane of the membrane as cross-linking of PMN CR1 by F(ab')$_2$ anti-CR1 resulted in an association of CR1 with the underlying cytoskeleton (Jack and Fearon 1983). In addition treatment of monocytes with colchicine resulted in the inhibition of the activation of CR1 which follows stimulation with fibronectin (Wright et al 1984). Therefore intact microtubules may be a prerequisite for activation of CR1 as well as for responding to chemotactic stimuli such as FMLP. Lummicolchicine is a functional analogue of colchicine, produced by irradiation of colchicine with longwave ultraviolet light. It has no measurable affinity for microtuble subunits and does not affect microtubule polymerisation. This drug had no effect on the FMLP stimulated increase (Fig 5.6) indicating that it is specifically disruption of microtubules which is responsible for the lack of response to FMLP. Similar results were found with cytochalasin B which inhibits polymerisation of microfilaments.

These results may indicate that there are 3 distinct pools of PMN CR1. One pool is expressed on the membrane at 0°C, a second is expressed at 37°C and does not require energy or an intact cytoskeleton for its expression, while a third pool which is expressed in the presence of chemotactic agents such as FMLP depends upon energy and an intact cytoskeleton for translocation to the membrane. Whether differentiation between pools 1 and 2 is of any value is questionable as PMN <u>in vivo</u> circulate at 37°C.

Further evidence for the existence of an internal pool of CR1 in PMN comes from the trypsinisation studies. It was found that trypsinised PMN

which were depleted of CR1 on the membrane surface, could regenerate CR1 following an incubation of 1 hour at 37°C (Fig 5.8; Table 5.6). The trypsinisation was carried out by incubation at 37°C for 15 min. Richerson et al (1985) studied the time course of temperature induced complement receptor enhancement. They found that warming to 37°C alone rapidly enhanced CR1 to nearly maximum levels within 5 min although further incubation up to 1 hour resulted in further slight enhancement. Thus during the trypsinisation process additional CR1 would have been translocated from an internal pool to the surface and removed by trypsin. Although this was the case, the pool of intracellular CR1 could not have been completely depleted as regeneration of CR1 did occur after incubation for 1 hour. Expression of this pool did not occur when the trypsinised PMN were held at 0°C indicating that the regeneration of CR1 was temperature dependent (Table 5.5). Cytochalasin B and colchicine did not affect regeneration of CR1 on trypsinised PMN (Fig 5.10) suggesting that an intact microfilament and microtubule system were not important for expression of this pool of CR1.

This observation lends further support to the theory that 2 intracellular pools of CR1 exist. The CR1 pool expressed after trypsinisation of PMN is different to that expressed after stimulation with FMLP as the former is not sensitive to inhibitors of cytoskeleton formation whereas the latter is. Further support for this may be provided by experiments in which trypsinised PMN are stimulated with FMLP to see if enhanced expression of CR1 over and above that seen at 37°C is observed and if this enhanced expression can be inhibited by cytochalasin B and colchicine.

It is now well established that patients with SLE have reduced E CR1 (Miyakawa et al 1981; Wilson et al 1982; Inada et al 1982; Iida et al 1982; Walport et al 1985; Ross et al 1985; Holme et al 1986). This deficiency is now known not to be restricted to E. Emancipator et al (1983) showed that patients with SLE proliferative nephritis had a deficiency of CR1 on their glomerular podocytes. Hurst, Nuki and Wallington (1984) reported a significant reduction in the rate of complement mediated phagocytosis by blood monocytes in patients with SLE. This reduction was most evident in patients with active disease. Wilson et al (1986b), evaluated the expression of CR1 on B cells by 2 colour fluorescent flow cytometry and found that the mean relative fluorescence in 17 SLE patients was 39% lower than in 17 normal individuals. They also studied the CR1 content of detergent lysates of patients neutrophils and found them to be 41% lower than normal. However they could not find any relationship between CR1 number and laboratory parameters of disease activity such as auto-anti-CR1, C4, C3 and immune complex levels.

I have found that all 3 pools of PMN CR1 were reduced in SLE patients with active disease (Fig 5.12). In fact there was no significant increase in PMN CR1 expression found by stimulation with FMLP in patients in an active state of disease over the spontaneous increase seen at 37°C (Fig

5.12). This may indicate that, (i) the FMLP CR1 pool in these patients is severely depleted or (ii) that these patients have a defect in the mechanism leading to translocation of CR1 or (iii) that there are a diminished number of chemotactic receptors for FMLP on the surface of the PMN from patients with SLE. If this last possibility was found to be true it may be that a CR1 deficiency is only one representation of a wider membrane defect on the PMN of these patients.

In SLE patients there were significant correlation coefficients between levels of E CR1 and levels of CR1 in each of the 3 PMN pools, (Fig 5.13(b), (c) and (d)). In addition there were also significant correlations coefficients between each of the 3 pools of PMN CR1, (Fig 5.14(a), (b) and (c). Before beginning this study on PMN it was not known if the reduction in CR1 on the E of patients with active SLE could be compensated by higher CR1 levels on their peripheral blood cells. The significant correlation coefficients between the differing pools of PMN CR1 and between E CR1 levels and PMN CR1 levels indicate that a deficiency in E CR1 is not compensated by increased PMN CR1 levels nor is a deficiency in surface expression of PMN CR1 compensated by larger internal pools of PMN CR1.

The finding that SLE patients in remission had increased CR1 expression in all 3 pools (Fig 5.12) and that PMN CR1 numbers rose when the disease entered phases of remission (Table 5.7) suggests that this abnormality is an acquired defect.

The role of CR1 on PMN in the process of phagocytosis is still unclear. Unstimulated PMN will not ingest sheep EAIgMC3b but require first to be activated by chemotactic factors such as FMLP or C5a des Arg followed by stimulation with PMA or fibronectin (Pommier et al 1984 and Changelian et al 1985). This acquisition of a phagocytic function correlates with phosphorylation of CR1 (Changelian and Fearon 1985) which may represent the structural basis for the activated state of the receptor. Both chemotactic factors and fibronectin are present at sites of tissue injury and they may represent an important *in vivo* mechanism for the augmentation of CR1 expression and activation at these sites. A reduced number of CR1 on the surface of PMN of patients with SLE and a depleted internal CR1 pool available for translocation may result in the inability of these cells to deal efficiently with the internalisation and degradation of immune complexes and the phagocytosis of opsonised bacteria. This in turn may predispose to bacterial infection a well known complication of SLE (Steinberg 1985) and also to the severity and duration of immune complex mediated tissue injury.

# CHAPTER SIX

## 6.1 Introduction

In the previous chapter it was shown that the PMN from patients with active SLE were not only deficient in surface expression of CR1 but the internal pools of CR1 were also depleted. One possible explanation for such a depletion could be that some abnormality exists in the biosynthesis of the receptor.

To date little is known of the biosynthesis of the receptor. One study on the human promyelocytic leukemia cell line has revealed that CR1 may be initially present as a 188,000 dalton intracellular precursor (Atkinson and Jones 1984); however no data exists on the synthesis, secretion and turnover of CR1 in cells from normal individuals and SLE patients. It was decided that as PMN are difficult to culture for long periods of time, to initially try to study the rate of synthesis of CR1 in cultured monocytes and lymphocytes from normal individuals. If this proved to be successful cells from patients with SLE could then be cultured and the rate of CR1 synthesis in these patients compared with normal individuals.

As there was no established method available for the immunoprecipitation of CR1 from $^{35}$S-methionine labelled monocytes, the optimal conditions for this technique had first to be characterised.

It was first necessary to optimise the culture conditions for protein synthesis by monocytes and lymphocytes before pulsing the cultures. In the case of monocytes the production of C2 was used as an indication that the monocytes were healthy and synthesising proteins. The stimulation of lymphocytes with mitogens ensured that the lymphocytes were synthesising an increased amount of protein. The immunoprecipitation of C3 from monocyte culture supernatants was an indication that the immunoprecipitation technique was reliable.

In this chapter I will present the methods used and the results obtained in attempting to establish optimal conditions for pulsing lymphocytes and monocytes and I will discuss the difficulties which were found in trying to immunoprecipitate CR1 from those cells.

Materials and Methods

## 6.2 Chemicals and other Reagents

Chemicals and other reagents were obtained from the following companies:

Gibco Biocult, Paisley, Renfrewshire

Antibiotic-Antimycotic Solution (100x) containing 10,000units Penicillin, 10,000μg Streptomycin and 25μg Fungizone
Basal Medium Eagle (BME) with Hepes (0.025 mol $l^{-1}$), without L-Glutamine
Dulbecco's Modified Eagles Medium (DMEM) with Sodium Bicarbonate, without Methionine, without L-Glutamine
Foetal Calf Serum (Sterile) (FCS)
Hanks Balanced Salt Solution (HBBS) without Sodium Bicarbonate
L-Glutamine (100x)
Sterile Nunclon Microtitre Plates (96 round bottomed wells with lid)
Sterile Nunclon Screw Cap Test Tubes (vol 13mls)

Sigma Chemical Company, Fancy Rd, Poole, Dorset

1,4 bis [2(4 methyl-5-phenyloxazolyl)] benzene (Dimethyl POPOP)
Deoxyribonucleic Acid (DNA) from Calf Thymus
L-Methionine
Phytolacca americana Mitogen (Pokeweed Mitogen (PWM))
Polyguanylic Acid (Poly G)

Flow Laboratories, Irvine, Ayrshire

Linbro (24 well plates 16mm diameter) Tissue Culture Plates
Titerteck Cell Harvester Filter Paper

Aldrich Chemical Company, Gillingham, Dorset

3'-5-Diaminobenzoic acid dibydrochloride (DABA)
2-5-Diphenyloxazole, Scintillation Grade (PPO)

Gallenkamp, East Kibride, Glasgow

G/Fibre Filter Circles (GF/C) 25mm
Whatman Grade 1 Filter Paper

Millipore UK Ltd, Millipore, Giysem,11-15 Peterborough Rd, Harrow, Middlesex

Millex-GS Sterile Filter Units (0.22μm pore size)

British Drug Houses (BDH) Chemicals Ltd, Poole, Dorset

Sodium Deoxycholate
Toluene
Triton X-100

May and Baker Ltd, Liverpool Rd, Barton Moss, Eccles, Manchester
Acetone
Methanol

James Burrough, 60 Montford Place, London

Ethanol

Pharmacy, Western Infirmary, Glasgow

Mannitol Injection BP 20%(w/v) in water

## 6.3 Monocyte Culture from Buffy Coat Blood Pack

### Reagents

#### Buffy Coats

Buffy coats from fresh (citrate phoshate dextrose anticoagulated) blood donations were supplied by the Blood Transfusion Service, Law Hospital, Carluke.

#### AB Plasma

AB plasma was prepared as previously described in chapter 5.9.

#### Foetal Calf Serum

Sterile foetal calf serum was heat inactivated at 56°C for 2 hours to destroy endogenous complement activity.

### Buffers and Solutions

#### Sodium Bicarbonate

A 7.5%(w/v) sodium bicarbonate solution was made up in deionised water. This solution was sterilised by passing through a Millex-GS filter.

#### Ficoll-Hypaque

Ficoll-Hypaque was prepared as previously described in Chapter 5.3.

Hanks Balanced Salt Solution (HBBS)

One packet of powdered HBBS was dissolved in 80mls of deionised water and 5mls of 7.5%(w/v) sodium bicarbonate were added. This solution was then made to 100mls with deionised water and filtered through a Millex-GS sterile filter into 900mls of sterilised deionised water.

RPMI

500mls of RPMI-1640 were supplemented with 5mls of antibiotic-antimycotic solution (100x), 5mls of L-glutamine (100x) and 5mls of 7.5%(w/v) sodium bicarbonate.

RPMI/AB

10mls of AB serum were added to 100mls of RPMI to give a 10%(w/v) solution.

RPMI/FCS

20mls of FCS were added to 100mls of RPMI to give a 20%(v/v) solution.

Procedure

Thirty millilitre aliquots of blood were centrifuged at 600g for 10 min at room temperature in sterile 55ml Falcon tubes and the plasma removed. The white cells were removed carefully from the top of the red cell pellet and made up to a volume of 40mls with HBBS. Twenty millilitres of this cell suspension were layered on to 15mls of Ficoll-Hypaque and centrifuged at 500g for 30 min at room temperature. The cells were collected from the interfacial layer and washed 4 times in HBBS. The first wash was for 10 min at 400g at room temperature and the remaining ones were washed for 5 min at 200g at room temperature. The cells were resuspended in RPMI containing 10% AB, counted in an improved Neubauer haemocytometer and standardised to $6x10^6$ cells/ml in the same medium. One third of the cells were plated directly into Linbro wells (500µl/well) and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After 30 min the non-adherent cells were removed from the wells by aspiration and the second third of the cells (which had been kept on ice for 15 min before warming to 37°C for 15min) were plated out onto the same Linbro wells at 500µl/well. This procedure was repeated for the last third of the cells which were kept on ice until 15 min prior to plating out where upon they were warmed to 37°C. After the final 30 min incubation the cells were washed gently 3 times in warm RPMI and finally cultured in 1ml of RPMI plus 10%AB. After 3 days in culture the culture medium was removed, the monolayer was washed 3 times in warm RPMI and cultured thereafter in 1ml of RPMI containing 20% FCS/well.

## 6.4 C2 Assay

### Reagents

#### EAC4 Intermediate

EAC4 intermediates were prepared by Mrs L Jones.

#### Human C1 and C2

Human C1 and C2 were prepared by Mrs L Jones.

#### Buffers and Solutions

#### D50S

50g of glucose plus 5.95g of sodium chloride were dissolved in 100mls of deionised water.

#### $GVB^{2+}$

$GVB^{2+}$ was prepared as previously described in Chapter 2.7(b).

#### $DGVB^{2+}$

$DGVB^{2+}$ was prepared as previously described in Chapter 2.7(b).

#### Mannitol $GVB^{2+}$

Three volumes of 20% mannitol solution were added to one volume of $GVB^{2+}$.

#### C-Rat

C-rat was prepared as previously described in Chapter 2.7(c).

#### Saline

Saline was prepared as previously described in Chapter 2.7(b).

### Procedure

To ensure that the monocytes were healthy and synthesising protein, 100µl samples of the culture supernatant were removed from each Linbro well on day 6 and day 11 of culture. One hundred microlitres of fresh culture medium was then added back to the wells. The removed culture samples were centrifuged for 2 min at 10,000g at room temperature to remove loose cellular debris and the supernatants were either stored at -70°C or transferred to an ice bath for immediate assay.

Frozen EAC4 intermediates were quickly thawed at 37°C in a shaking water bath and reconstituted according to the procedure shown below:

| Additions | Volume (ml) | Length of Incubation (min) |
|---|---|---|
| D50S | 0.23 | 5 |
| Mannitol $GVB^{2+}$ | 1.0 | 2 |
| Mannitol $GVB^{2+}$ | 2.0 | 2 |
| Mannitol $GVB^{2+}$ | 8.0 | 5 |
| $DGVB^{2+}$ | 5.0 | 2 |
| $DGVB^{2+}$ | 10.0 | 2 |
| $DGVB^{2+}$ | 10.0 | 2 |
| $DGVB^{2+}$ | 10.0 | 5 |

All additions were made dropwise while the cells were being shaken constantly. After the final addition of $DGVB^{2+}$ the cells were centrifuged at 2,000g for 10min at 2°C. The cell pellet was washed 3 times in $DGVB^{2+}$, resuspended to $1x10^8$/ml in $DGVB^{2+}$ and warmed to 30°C in a shaking water bath. An equal volume of functionally purified human C1 in $DGVB^{2+}$ (100units/ml) was pre-warmed to 30°C and added to the EAC4 with constant shaking. The cells were incubated at 30°C for 15 min before centrifugation at 200g for 5 min at 20°C. The cell pellet was resuspended to $1x10^8$/ml in $DGVB^{2+}$ and warmed to 30°C.

Twenty five microlitre aliquots of the supernatant from the monocyte cultures were added to a series of test tubes containing 100µl of $DGVB^{2+}$ and the tubes were warmed to 30°C. One hundred microlitres of EAC14 were added to each tube and incubated for a pre-determined T max of 2½ min after which 300µl of C-rat were added and the tubes incubated at 37°C for 1 hour in a shaking water bath for lysis to occur. The reaction was stopped by addition of 2mls of saline to all tubes except the 100% lysis control, to which 3mls of water were added. The tubes were centrifuged at 2,000g for 5 min at 4°C and the $OD_{414}$ of the supernatant was measured.

A series of control tubes containing the additives shown below were measured along with the culture samples.

| Control Tubes | Reagents Added to the Control Tubes | | |
|---|---|---|---|
| | $DGVB^{2+}$µl | EAC14µl | C-Ratµl |
| Cell Blank (CB) | 400 | 100 | - |
| Reagent Blank (RB) | 100 | 100 | 300 |
| 100% Lysis | 100 | 100 | 300 |
| Complement Colour (CC) | 200 | - | 300 |

A standard C2 curve was also prepared by doubly diluting purified C2 of a known number of effective units/ml, in $DGVB^{2+}$. One hundred microlitre aliquots of these dilutions (1/250-1/8000) were added to separate test tubes and the tubes were treated in the same manner as the samples, this providing a positive control for the assay.

Calculation of Results

The results were calculated according to the formula:

$$Y = \frac{\text{OD sample} - \text{OD}_{\text{RSB}}}{\text{OD 100\% lysis} - \text{OD}_{\text{RSB}}}$$

Percent lysis = 100 x Y
The number of haemolytic units (Z) was calculated using the formula:

$$Z = -\ln (1-Y)$$

The em of C2/ml of culture fluid for a 25µl sample (corrected for the number of EAC4 added to each tube =

$$Z \times \frac{1000\mu l}{25\mu l} \times 10^7$$

The em of C2/well are expressed per microgram of DNA, therefore:

$$\frac{\text{em of C2/well}}{\mu g \text{ DNA/well}} = \text{C2 em/well/}\mu g \text{ DNA}$$

6.5 DNA Assay

Buffers and Solutions

2%(w/v) SDS

2g of SDS were added to 100mls of deionised water.

Tris (0.1 mol $l^{-1}$) EDTA pH 7.5

121g of Tris were dissolved in 10mls of deionised water and the pH adjusted to 7.5 using concentrated HCl. 57.5mls of EDTA (0.086 mol $l^{-1}$) (prepared as described in Chapter 2.7(b)) were added and the solution was made up to 1 litre with deionised water.

Polyguanylic Acid (Poly G) Solution

10mg of Poly G were added to 10mls of Tris (0.1 $mol^{-1}$) EDTA pH 7.5.

Deoxyribonucleic Acid (DNA) Solution

DNA from calf thymus was made up to a 1mg/ml stock solution in 2%(w/v) SDS and diluted as appropriate.

Procedure

In order to quantify the number of adherent monocytes in the Linbro wells the culture medium was removed from 6 sample wells and the monolayer washed gently with warm RPMI to remove debris and nonadherent cells. One millilitre of SDS(2%(w/v) in water) was added to each of the wells and the plate incubated for 1 hour at 37°C. The cell lysates were removed and 460µl of each were transferred to microcap tubes . To precipitate the DNA from these cell lysates, 10µl of poly-G solution and 900µg of ice-cold ethanol were added to the tubes which were vortexed and stored for 18 hours at -20°C. Upon thawing, the microcap tubes were centrifuged at 900g for 5 min at room temperature, the supernatants were discarded and the pellets washed 3 times in 500µl of absolute ethanol and dried for 30 min at 100°C. Fifty microlitres of deionised water were added to each pellet, which was then resuspended and incubated at room temperature for 30 min. Twenty five microlitres of diaminobenzoic dibydrochloride (DABA) an agent which fluoresces when bound to DNA was added, the tubes were vortexed and incubated for a further 30 min at 60°C after which 500µl of hydrogen chloride (1 mol $l^{-1}$) were added to solubilise the pellet. The tubes were centrifuged at 9,000g for 5 min at room temperature and the supernatants were then read using a Perkin Elmer 100 Spectrofluorimeter with excitation at 400nm and emission at 500nm.

A standard DNA curve was constructed by preparing samples containing 5, 2.5, 1.25, 0.625 and 0.312µg of DNA/ml in 460µl of 2%(w/v) SDS. These samples plus a blank containing only 460µl of 2%(w/v) SDS were treated in the same way as described above. The fluorimeter was zeroed with the blank and the standard containing 5µg/ml DNA was read on the fluorimeter. This standard was used to calibrate the machine and once calibrated the concentration of DNA/ml in the remaining standards and samples were read directly from the fluorimeter. The number of cells per well could be estimated from the DNA content, 1 to 1.5µg of DNA being equivalent to $1x10^5$ monocytes.

## 6.6 Internal Labelling of Monocyte Proteins with $^{35}$S-Methionine

### Buffers and Solutions

### Dulbecco's Modified Eagles Medium (DMEM) (Methionine-Free)

100mls of DMEM without methionine, without sodium bicarbonate and with L-glutamine were supplemented with 1ml of antibiotic antimycotic solution (100x) and 1ml of L-glutamine (100x).

### DMEM (Containing Methionine)

30μg of methionine were added to 10mls of methionine-free DMEM supplemented with antibiotic antimycotic solution and L-glutamine as before.

### Lysis Buffer

PBS (prepared as described in Chapter 2.6(e)) containing 1%(v/v) NP-40 0.5%(w/v) SDS, EDTA (3 mmol $l^{-1}$) and PMSF (2 mmol $l^{-1}$).

### $^{35}$S-Methionine

$^{35}$S-Methionine was made to a concentration of 500 μCi/ml in methionine-free DMEM.

### Procedure

The culture fluid was removed from each Linbro well and the monocyte monolayers were washed 3 times with warm methionine-free DMEM. Two hundred microlitres of the same medium were then added to each well and the monolayers were incubated at 37°C for 15 min. The medium was removed and 333μl of $^{35}$S-methionine were added. The monocytes were incubated for the desired pulse times at 37°C in a humidified atmosphere of 5% $CO_2$ in air. When the pulse was complete the supernatant was removed from each well, placed into a microcap and centrifuged at 10,000g for 5 min at room temperature to remove cellular debris. The supernatant was placed into a fresh microcap and 5μl of PMSF (1 mol $l^{-1}$) and 5μl of EDTA (0.086 mol $l^{-1}$) were added. These proteases were added to prevent proteolysis of soluble CR1 which may have been present in the supernatant.

The cells in the monolayer were washed once in methionine-free DMEM and then incubated in DMEM containing methionine (5 μg/ml) to "chase" the $^{35}$S-methionine through the protein synthetic pathway. Once the chase was completed the supernatant was removed and treated in a similar way to that described previously. The remaining monocytes were lysed by incubation for 1 hour at 37°C in *1ml* of lysis buffer. The lysis buffer was then removed and placed into microcaps. These were

centrifuged at 10,000g for 5 min at room temperature to remove the insoluble membranes. The lysates containing solubilised proteins were transferred to fresh microcaps and protease inhibitors were added as before. The lysates were stored at -70°C.

6.7 Determination of $^{35}$S-Methionine Incorporation into Cell Lysates and Supernatants using the Filter TCA Precipitation Method.

Reagents

GF/C circle filters were soaked in methionine (1 mmol $l^{-1}$) and dried overnight at room temperature.

Solutions

Methionine (1 mmol $l^{-1}$)

A 1 mmol $l^{-1}$ methionine solution was made up in deionised water.

TCA Solution

A stock solution of 20%(w/v) TCA in deionised water was prepared and diluted as appropriate.

Acetone/Ethanol Solution

An equal volume of acetone and ethanol were mixed together.

Scintillation Fluid

0.18 of 1.4 bis [2 (4 methyl-5-phenyloxazolyl) ] benzene (POPOP) and 3g of 2-5 Di-phenyloxazole (PPO) were dissolved in 600mls of toluene at 56°C, 300mls of Triton X-100 were then added and the solution was mixed thoroughly.

Procedure

In order to determine the amount of radioactive methionine which had been incorporated into the cellular proteins during the pulse-chase experiments, 5µl of the supernatant or lysate were spotted onto the pre-soaked filters. These were then dried in an oven at 80°C. The filters were then given a series of 1 min washes in each of the following solutions, (i) 10% TCA, (ii) 5% TCA, (iii) acetone, (iv) acetone/ethanol solution and (v) 100% ethanol. These washes result in the precipitation of protein including that which has incorporated $^{35}$S-methionine. This protein remains bound to the filter while free $^{35}$S-methionine is washed away. After the final wash in ethanol, the filters were dried in an oven and then placed into scintillation vials containing 5mls of scintillation fluid. The amount of incorporated $^{35}$S-methionine was then determined by

counting the scintillation vials in a β counter. The efficiency of incorporation was determined by the following equation:

$$\frac{\text{Total cpm in the lysate}}{\text{Total cpm input}} \times 100$$

## 6.8 Viability of Monocytes in Methionine-Free DMEM

### Solutions

#### Trypan Blue (0.75(w/v))

Trypan blue solution was prepared as described in chapter 5.7.

#### Normal Saline

Normal saline was prepared as described in chapter 2.7(b).

### Procedure

In order to assess how long the monocytes could be pulsed in methionine-free DMEM while still remaining viable the following experiment was performed. The culture medium was removed from 8 wells of a monocyte culture. To 7 of these wells 100μl of $^{35}$S-methionine (500μl/ml) were added after washing the monolayers once with methionine-free DMEM. Trypan blue (200μl) was then added to the eighth well which was the control and the cells were incubated for 2 min. After removal of excess trypan blue by aspiration the viability of the monocytes was assessed by observing under a light microscope the number of cells which had excluded trypan blue. The wells to which $^{35}$S-methionine had been added were pulsed for a variety of time intervals, 1 hour, 2 hours, 3 hours, 4 hours and 6 hours. At the end of each time interval the medium was removed and 200μl of trypan blue were added to the wells. The viability of the monolayer was then assessed by trypan blue exclusion.

## 6.9 Immunoprecipitation of C3 from Monocyte Supernatants

### Reagents

#### Anti-C3 Antiserum and Anti-BSA Antiserum

Anti-C3 antiserum and anti-BSA antiserum were donated by Mr A Hamilton. These were diluted 1:10 in PBS for use.

Protein A Cell Suspension

A protein A cell suspension was prepared as described in chapter 4.14(a).

Procedure

Monocytes are known to synthesis C3 in nanogram quantities,( Strunk, Kunke and Giclas 1983). It was therefore decided to immunoprecipitate this protein from the supernatants of monocytes which had been pulsed with $^{35}$S-methionine to establish that the immunoprecipitation technique was reliable.

One millilitre of a monocyte supernatant from a monolayer which had been pulsed for 2 hours was absorbed with 25µl of a protein A cell suspension as was described in chapter 4.14(b). The supernatant was then divided equally between 2 microcap tubes. To 1 of these microcaps 5µl of a 1:10 dilution of anti-C3 antiserum was added and 5µl of a 1:10 dilution of anti-BSA antiserum was added to the second microcap tube. These tubes were rotated end over end on a Matburn mixer for 1 hour at room temperature after which 25µl of a protein A cell suspension was added and the incubation continued for 30 min at room temperature. The proteins which had bound to the protein A cell suspension were eluted as described in chapter 4.14(b).

## 6.10 Immunoprecipitation of CR1 from Monocytes

Reagents

Anti-CR1

A commercially available monoclonal anti-CR1 antibody was utilised.

Normal Mouse Serum (NMS)

NMS was supplied by the Animal Laboratory, Glasgow University.

Procedure

The monocyte lysate (1ml) was absorbed with 25µl of a protein A cell suspension. The lysate was then divided equally between 2 microcap tubes. To 1 of these tubes 5µl of neat mouse anti-CR1 ascites fluid were added and 5µl of NMS were added to the other. These tubes were rotated end over end on a Matburn mixer for 1 hour at 4°C after which 5µl of the second antibody rabbit anti-mouse IgG was added to both tubes and the incubation continued for 1 hour at 4°C. Following this, 25µl of a protein A cell suspension were added and the tubes were incubated for a further 30 min at 4°C. The proteins which had bound to the protein A cell suspension were eluted as previously described in chapter 4.14(b).

CR1 was immunoprecipitated from monocyte supernatants using an identical procedure to that described for the lysates.

6.11 SDS-PAGE

Samples were electrophoresed on 5 and 7½% slab gels as described in chapter 2.6(h) and then subjected to fluorography.

6.12 Fluorography

Solutions

Destain Solution

Destain solution was prepared as previously described in chapter 2.6(f).

PPO-DMSO (24%(w/v))

24g of 2-5 diphenyloxazole (PPO) were dissolved in 100mls of pre-heated DMSO.

Glycerol Solution

2.4mls of glycerol were mixed with 97.6mls of deionised water.

Procedure

The slab gel was fixed for 30 min in destain and then given 2 500mls washes in DMSO for 30 min each. This was removed and the gel was incubated in 200mls of PPO-DMSO solution for 45 min after which the gel was washed in deionised water for 1 to 2 hours. Finally the gel was soaked in glycerol solution for 30 min to prevent cracking.

The gel was transferred to a sheet of Whatman No 1 filter paper and placed on a drying board. The board was sealed with cellophane and the gel dried overnight under vacuum on a freeze drier.

The dried gel was then placed into a X-ray cassette in contact with a sheet of X Ray film and placed at -70°C for the required time. The exposure time required was estimated from the $^{35}S$-methionine incorporation into the sample put onto the gel. A period of 2 weeks was usually sufficient to allow development of bands containing more than 3,000cpm. The film was then developed as previously described in chapter 4.14(b).

6.13 Lymphocyte Culture from 60mls of Blood

Buffers and Solutions

Sterile Saline

Saline was prepared as described previously in Chapter 2.7(b). This was then filtered through a Millex-GS sterile filter unit.

Ficoll-Metrizoate

30mls of sodium metrizoate 32%(w/v) were mixed with 72mls of Ficoll 8%(w/v) (SG 1.077).

Basal Medium Eagle (BME)

BME with Hepes (0.025 mol $l^{-1}$).

RPMI/AB

RPMI was supplemented as previously described in (6.3) and 5%(v/v) AB serum was added.

Procedure

60mls of venous blood were collected into 3 universal containers containing 300µl of sodium heparin (1000 units/ml). The blood was then placed into a beaker and diluted 1 in 2 with sterile saline and 15ml aliquots were layered over 10mls of Ficoll-Metrizoate in sterile universal containers. The blood was centrifuged at 400g for 30 min at room temperature after which the white interfacial layer composed of mononuclear cells was removed and placed into a universal container. The cells were made up to a volume of 20mls in BME and centrifuged at 200g for 30 min at room temperature. The supernatant was discarded and the cell pellet washed by resuspension in BME and centrifugation at 200g for 10 min at room temperature and then at 200g for 5 min at room temperature. The cells were resuspended in 20mls of RPMI. A 10µl aliquot of the cell suspension was diluted with 190µl of white cell counting fluid, the mononuclear cells were counted in an improved Neubauer haemocytometer and standardised to $1x10^6$/ml in RPMI/AB. Five millilitres were added into sterile culture test tubes and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

## 6.14 Stimulation of Lymphocytes with Pokeweed Mitogen (PWM)

Reagents

PWM

PWM was stored at -20°C as a stock solution of 1mg/ml in RPMI. It was then diluted as appropriate.

$^{3}$H-Thymidine

$^{3}$H-thymidine was supplied by the Radioisotope Dispensary, Western Infirmary Glasgow. It was stored at 4°C as a 10μCi/ml solution in RPMI.

Scintillation Fluid

Scintillation fluid was prepared as described earlier (6.7).

Procedure

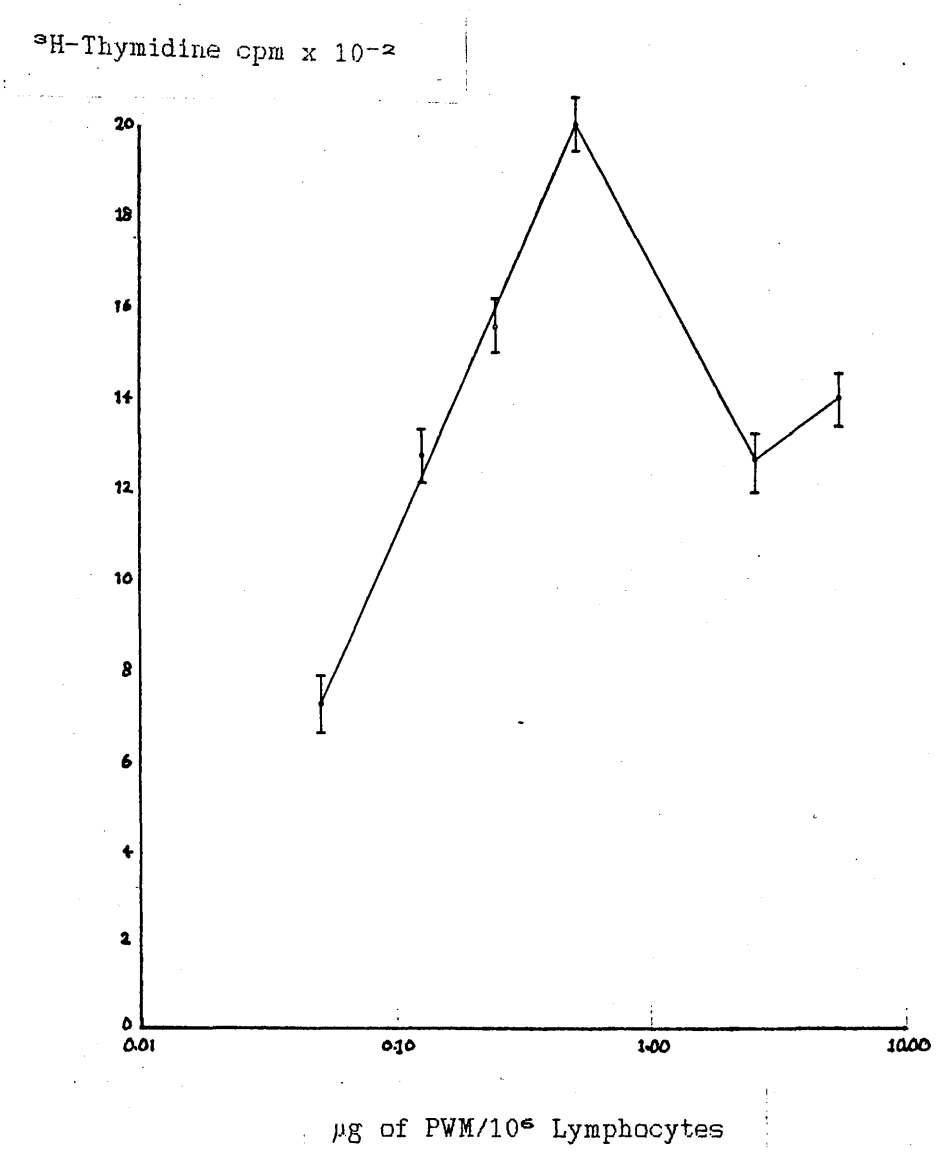
Lymphocytes (1x10$^{6}$/ml) in RPMI/AB were plated out into sterile microtitre plates (200μl/well). Ten microlitres of a range of concentrations of PWM (1.25-500ug/ml) were then added in triplicate to the wells, leaving 6 wells as controls. The cells were incubated for 48 hours at 37°C in a humidified atmosphere of 5% $CO_2$ in air. On the third day the lymphocytes were pulsed by the addition of 0.1μCi $^{3}$H-thymidine and incubation continued for 12 hours. The cells were harvested onto filter papers using a 12-well multimesh cell harvester. The harvesting process consisted of a series of washes in deionised water followed by 5% TCA and finally by methanol. The filters were dried at 37°C for 30 min, each filter was then placed into a vial containing 5mls of scintillation fluid and counted in a liquid scintillation β counter. A plot of the PWM concentration per 1x10$^{6}$ cells against the number of cpm was then drawn (Fig 6.1). As a concentration of 0.5μg/1x10$^{6}$ cells resulted in the greatest amount of $^{3}$H-thymidine incorporation, this was used in all subsequent lymphocyte transformations.

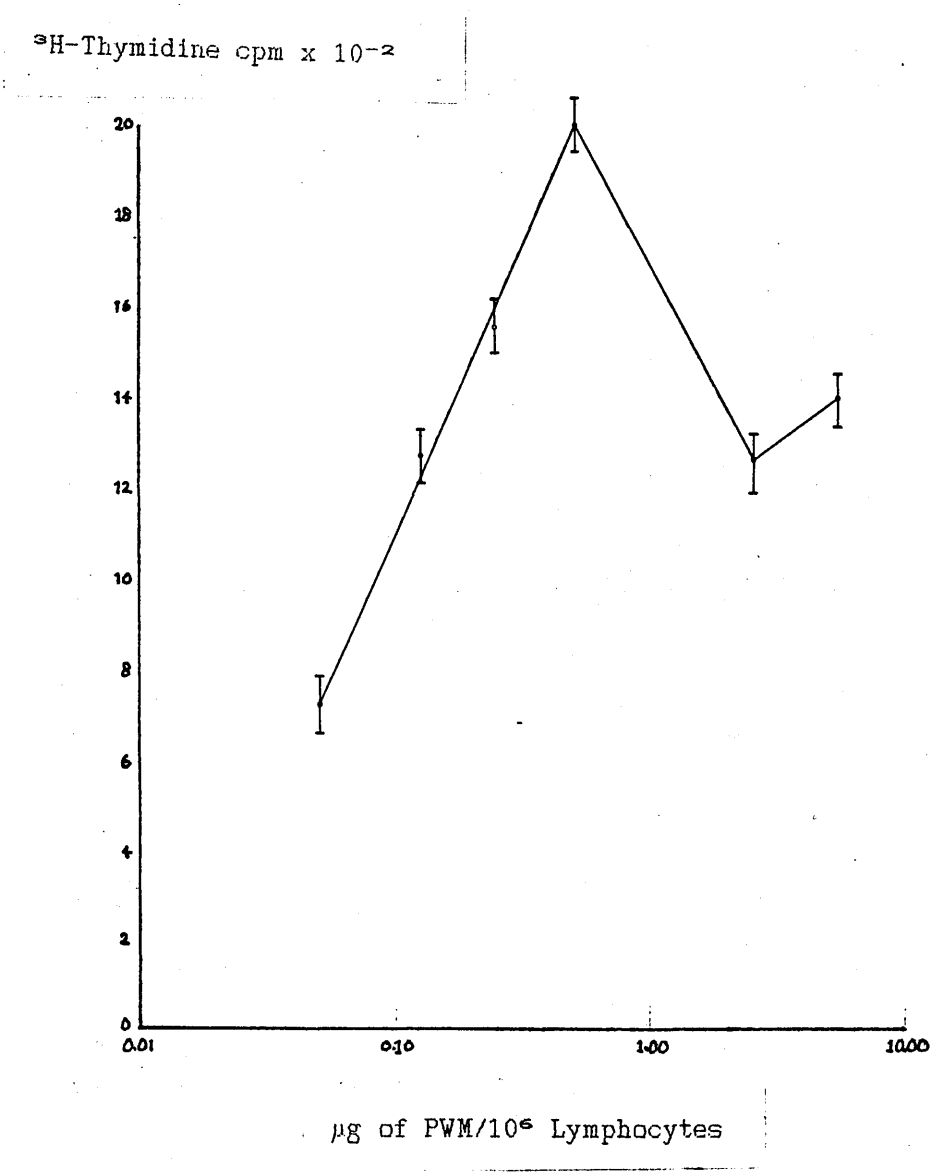
Internal Labelling of Lymphocyte Proteins with$^{3}$H-Thymidine

Before a lymphocyte culture was pulsed it was first of all established that the cells had been stimulated by the PWM. This was accomplished by setting up a microtitre plate containing lymphocytes (1x10$^{6}$/ml, 200μl/well) at the same time as the lymphocyte cultures were set up. The lymphocytes in the microtitre plate were treated with PWM (0.5μg/1x10$^{6}$ cells) and pulsed with $^{3}$H-thymidine on the third day. The incorporation of $^{3}$H-thymidine into these cells indicated whether the cells had been stimulated or not. If they had, then it was assumed that the lymphocytes in culture had also been stimulated and the culture was then pulsed with $^{35}$S-methionine.

6.15 Internal Labelling of Lymphocyte Proteins with $^{35}$S-Methionine

Sterile culture test tubes containing 5x10$^{6}$ lymphocytes which had been stimulated with 0.5μg of PWM/1x10$^{6}$ cells were centrifuged at 400g for 5 min at room temperature. The supernatant was carefully removed and the cells were resuspended in 500μl of warm methionine-free DMEM and incubated for 15 min. The cells were centrifuged at 400g for 5 min at room temperature, the supernatant was removed and the cells were


$^{3}$H-Thymidine cpm x $10^{-2}$
20
18
16
14
12
10
8
6
4
2
0
0.01
0.10
1.00
10.00
µg of PWM/$10^{6}$ Lymphocytes


$^{3}$H-Thymidine cpm x $10^{-2}$
20
18
16
14
12
10
8
6
4
2
0
0.01
0.10
1.00
10.00
µg of PWM/$10^{6}$ Lymphocytes

resuspended in 400µl of the above medium. One hundred microlitres of $^{35}$S-methionine (1mCi/ml) were added to each tube and the cells were left to incubate for the desired pulse time.

Once the pulse time was completed the cells were washed twice in DMEM containing methionine (3µg/ml), resuspended in 500µl of this medium and incubated at 37°C for the appropriate chase time. When the chase period was completed the supernatant was removed and placed into a microcap tube which was then centrifuged at 10,000g for 5 min to remove any cell debris. The supernatant was removed and 5µl of PMSF (1 mmol $l^{-1}$) and 5µl of EDTA (0.086 mol $l^{-1}$) were added and this was stored at -70°C. The cell pellets were resuspended in 500µl of lysis buffer and the lysates were mixed overnight at 4°C to allow solubilisation of CR1, finally they were centrifuged at 10,000g for 2 min at room temperature to remove the membranes. The clear lysate was then stored at -70°C.

## 6.16 Immunoprecipitation of CR1 from Lymphocyte Lysates and Supernatants

An identical protocol to that described earlier for monocytes was used (6.10). However, as the lymphocytes lysates and supernatants were in a total volume of 500µl and not 1ml which was the case for monocytes, a separate sample was used for the control and specific precipitation instead of dividing the lysates and supernatants after the first absorbtion with the protein A cell suspension.

## 6.17 Isolation of CR1 from Lymphocytes Lysates by Chromatography

As an alternative means of isolating CR1 from the lysates of lymphocytes it was decided to try a series of purifications steps on a Biorex column and a C3-Sepharose column.

### Chromatographic Material

### Biorex Column

A 1ml Biorex column was poured in a 2ml syringe barrel. This column was equilibrated with Biorex equilibration buffer as described in Chapter 2.9(a).

### C3-Sepharose

C3-Sepharose was prepared as previously described in Chapter 2.9(b).

### IgG-Sepharose

IgG-Sepharose was prepared by Dr W Mitchel.

Buffers

Borate Buffer

Borate buffer was prepared as described in Chapter 2.9(b) and sodium chloride (0.05 mol $l^{-1}$) and NP-40 1%(v/v) were then added.

Acetic Acid Solution

Acetic acid (0.1 mol $l^{-1}$) containing sodium chloride (0.4 mol $l^{-1}$) and NP-40 1%(v/v).

Neutralisation Buffer

Tris HCl (2 mol $l^{-1}$ pH 8.6) containing 1% NP-40.

Procedure

Fourteen test tubes containing $5x10^6$ lymphocytes were pulsed separately for 2 hours and chased for 1 hour. Five hundred microlitres of lysis buffer were then added to each cell pellet and the cells were stirred on a magnetic stirrer overnight at 4°C. The lysates were then pooled and centrifuged at 10,000g for 10 min at room temperature to remove the membranes. Sufficient deionised water to lower the conductivity to below 6mS at 0°C was added to the lysate before it was put over the Biorex column. The column was washed with 10mls of equilibrating buffer and CR1 was eluted with equilibrating buffer containing sodium chloride (0.6 mol $l^{-1}$).

Fractions (1ml) were collected and the column was screened by measurement of TCA precipitable protein as described previously (6.7). The fractions from the Biorex column which contained the highest incorporation of $^{35}S$-methionine were pooled and preabsorbed with 25µl of a protein A cell suspension and then divided equally between 2 tubes. One hundred microlitres of C3-Sepharose were added to 1 tube and 100µl of IgG-Sepharose were added to the remaining tube. Both were mixed for 2 hours at 4°C and both pellets were then collected by centrifugation at 10,000g for 2 min at room temperature. The pellets were washed 4 times in borate buffer and bound protein was eluted by 10 washes of 40µl each with acetic acid solution. Each 40µl was neutralised by addition of 5µl of neutralisation buffer.

A 5µl sample was measured for TCA precipitable counts and the remaining 40µl were mixed with sample buffer and analysed by SDS-PAGE.

Results

6.18 Synthesis of C2 by Monocyte Cultures

Haemolytic assays for C2 were performed on the monocyte culture supernatants to establish that the cells were synthesising protein before exposing them to $^{35}$S-methionine. The mean amount of C2 production from 6 wells of a sample culture after 6 days synthesis was 42±12x10$^{7}$ effective molecules (em) of C2 per well and the DNA content of these wells was 1.4μg/ml. Thus after 6 days there were 30±8.5x10$^{7}$ em of C2 per μg of DNA in each well. This was comparable to that found by Einstein, Schneeberg and Colten (1976). The culture was incubated for a further 5 days and the C2 content was measured again after 11 days synthesis and found to be 39±6x10$^{7}$ em C2/μg DNA/well. It was therefore decided to measure the C2 production of all subsequent cultures on day 6 and if this production was normal they would be pulsed with $^{35}$S-methionine on day 7.

6.19 Pulse-Chase Study in Monocytes

As shown below the viability of the monocytes as assessed by trypan blue exclusion decreased after the monocytes had been pulsed for 2 hours in methionine-free DMEM containing $^{35}$S-methionine.

| Length of Pulse (hours) | 0 | 1 | 2 | 4 |
|---|---|---|---|---|
| Percentage Viability | 100 | 100 | 100 | 64 |

It was therefore decided to pulse 7 day old monocytes for a maximum of 2 hours and to chase them for up to 2 hours in DMEM containing methionine. Incorporation of $^{35}$S-methionine was then measured in both the cell lysates and the culture supernatants. As might be expected, with increasing chase time the TCA precipitable protein in the cell lysate fell while that of the supernatant increased, indicating that radiolabelled proteins were being secreted from the monocytes into the surrounding culture medium (Fig 6.2).

Having established a method which resulted in the incorporation of between 5% and 12% of the added radioactive methionine into cellular proteins it was decided to immunoprecipitate the lysates and supernatants for C3 and CR1.

6.20 Immunoprecipitation of C3

Immunoprecipitation of C3 from the supernatant of a culture which had been pulsed for 2 hours revealed 2 specific bands of molecular weight 116,000 daltons and 75,000 daltons (Plate 6.1), which represent the α

Fig 6.2 Pulse-Chase Study in Monocytes

Legend

Five microlitres of supernatant or lysate from a monocyte culture, which had been pulsed for 2 hours and chased for up to 240 min, were spotted onto filter papers pre-soaked with methionine (1 mmol $l^{-1}$) and the amount of $^{35}S$-methionine incorporation was determined using the filter TCA protein precipitation technique. The number of cpm in each was then plotted against the chase time. As can be seen from the graph with increasing length of chase time the TCA precipitable protein in the cell lysate fell (_____), while that in the supernatant increased (- - -).

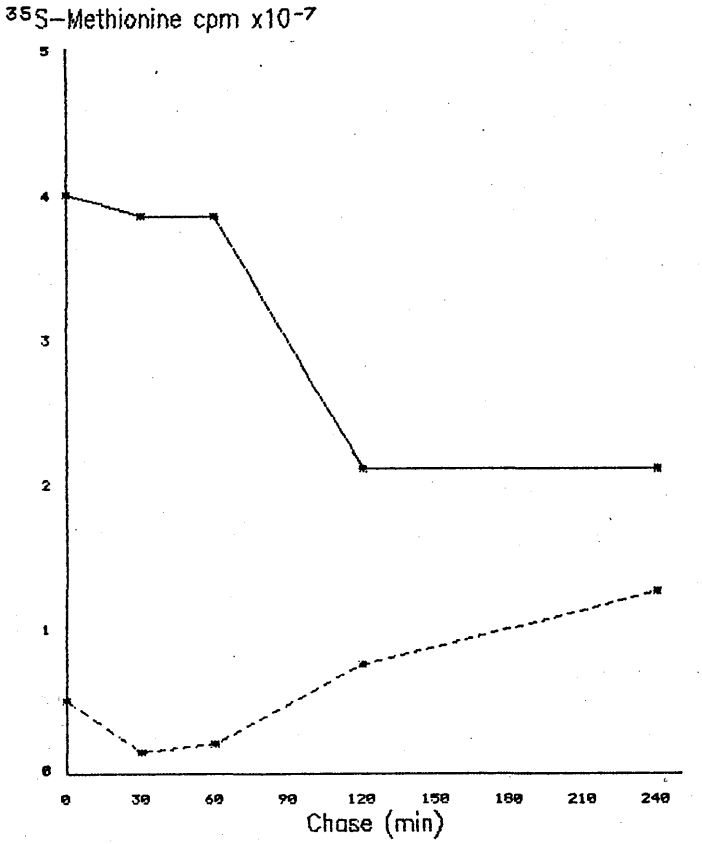
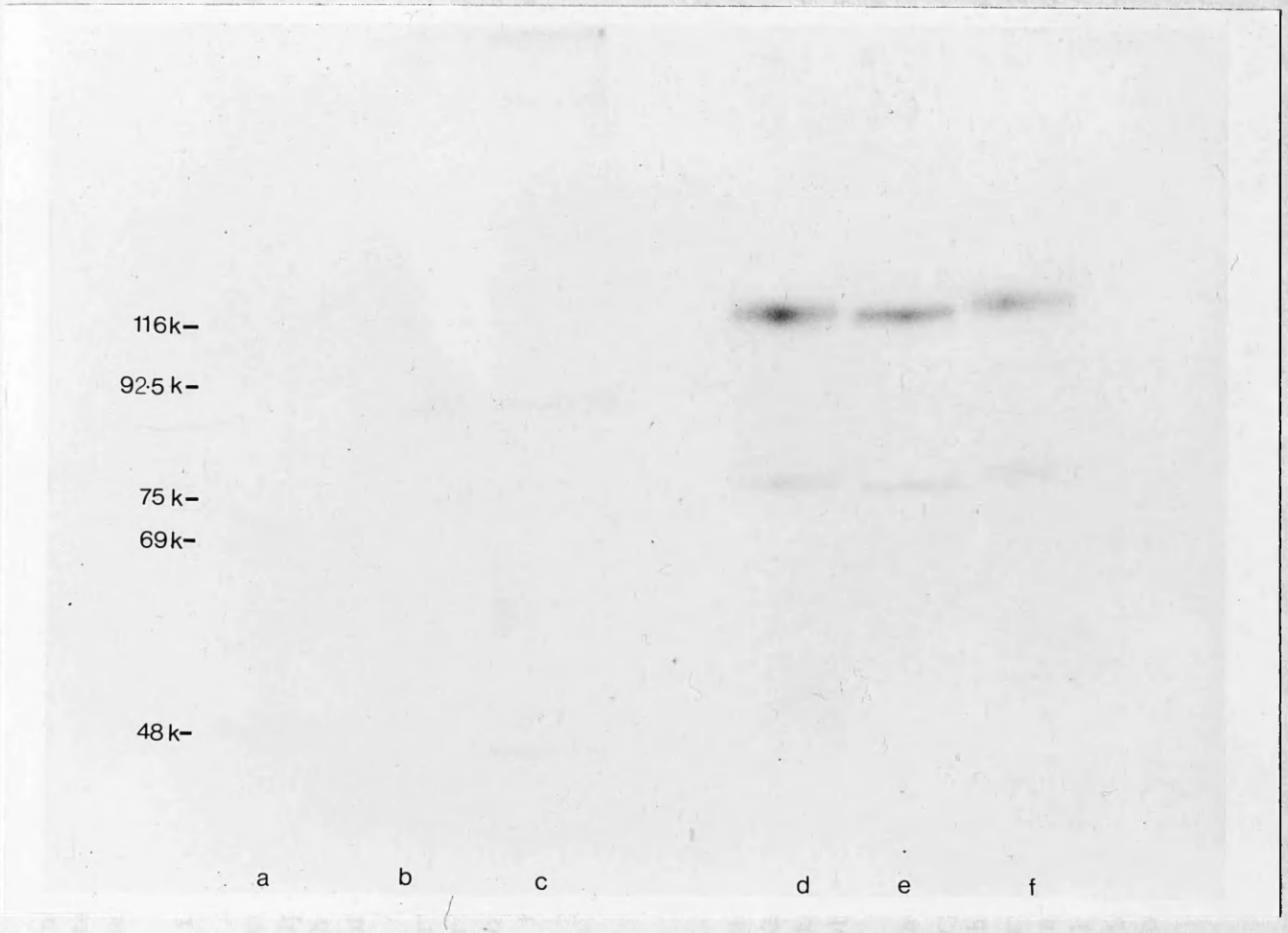

$^{35}$S–Methionine cpm x10$^{-7}$
5
4
3
2
1
0
0
30
60
90
120
150
180
210
240
Chase (min)

Plate 6.1 Immunoprecipitation of C3 from Monocyte Culture Supernatants

Monocyte culture supernatants were immunoprecipitated with anti-C3 antiserum. Samples were then analysed by SDS-PAGE on a 7½% slab gel run under reducing conditions. Tracks d, e and f show purified radiolabelled C3, track b shows the sample from the culture supernatant immunoprecipitated with anti-C3 antiserum and track c shows the control sample immunoprecipitated with anti-BSA. The α and β chains of C3 with molecular weights of 116,000 and 75,000 daltons can be seen in tracks d, e and f and very faintly in track b. No specific bands were seen in the control track.


116k–
92·5 k–
75 k–
69k–
48 k–
a
b
c
d
e
f

and β chains of C3. This established that the immunoprecipitation method was working.

6.21 Immunoprecipitation of CR1 from Monocyte Supernatants and Lysates

Immunoprecipitation of CR1 was performed for each supernatant and lysate of a monocyte culture which had been pulsed for 2 hours and chased for 15, 30, 60, 120, 240 and 360 min.

The immunoprecipitation samples were subjected to SDS-PAGE on 5% slab gels followed by fluorography. Plate 6.2 shows that a specific band of molecular weight 230,000 daltons was seen in each of the lysates precipitated with anti-CR1. This band was not seen in the controls which had been immuno-precipitated with NMS. Precipitation of the culture supernatant did not result in any specific bands.

Unfortunately, the precipitation of CR1 from 8 subsequent pulse-chase experiments did not result in any obvious specific bands apart from in one other case where a specific band of molecular weight 225,000 daltons was immunoprecipitated by anti-CR1 from a monocyte culture which had been pulsed for 3 hours with no chase (Plate 6.3).

6.22 Mitogenic Stimulation of Lymphocytes

When a mitogen such as PWM binds to T and B lymphocytes the cells are stimulated to increase protein synthesis and to undergo cell division. Lymphocyte cultures were therefore treated with PWM to ensure that synthesis of protein by the lymphocytes would be maximised. The degree to which the lymphocytes were stimulated was determined by measuring the amount of $^{3}H$ - thymidine which was incorporated into newly synthesised DNA. Lymphocytes which had been stimulated with PWM incorporated more than 30-fold more $^{3}H$ - thymidine than unstimulated lymphocytes. Prior to pulsing lymphocyte cultures with $^{35}S$-methionine, incorporation of $^{3}H$-thymidine was measured in the test microtitre plate set up at the same time as the culture, if transformation had occured the lymphocytes in the sterile culture test tubes were pulsed with $^{35}S$-methionine. Pulsing PWM stimulated lymphocytes resulted in a 6-fold greater incorporation of $^{35}S$-methionine into the cells (Fig 6.3).

6.23 Optimal Culture Conditions for Pulsing Lymphocytes

Pulse-chase studies were performed on stimulated lymphocytes on day 1, 3 and 5 in culture in order to determine which period of culture resulted in maximum incorporation of $^{35}S$-methionine. The maximum percentage of $^{35}S$-methionine incorporation into the lysates of 3 lymphocyte cultures which had been given a 2 hour pulse followed by a 2 hour chase was on day 3 in culture.

Plate 6.2 Immunoprecipitation of CR1 from Monocyte Lysates

CR1 was immunoprecipitated from the cell lysates of a monocyte culture which had been pulsed for 2 hours and chased for 15 min track a, 30 min track b, 60 min track c, 120 min track d, 240 min track e and 360 min track f. Tracks h and i represent the control precipitations with NMS and track g the HMW markers. Samples were analysed on a 5% slab gel run under reducing conditions. A specific band of molecular weight 230,000 daltons was immunoprecipitated from lysates which had been chased for 15 min, 30 min, 60 min, 120 min and 240 min. No specific band was seen in the lysate chased for 360 min or in the 2 control precipitations.

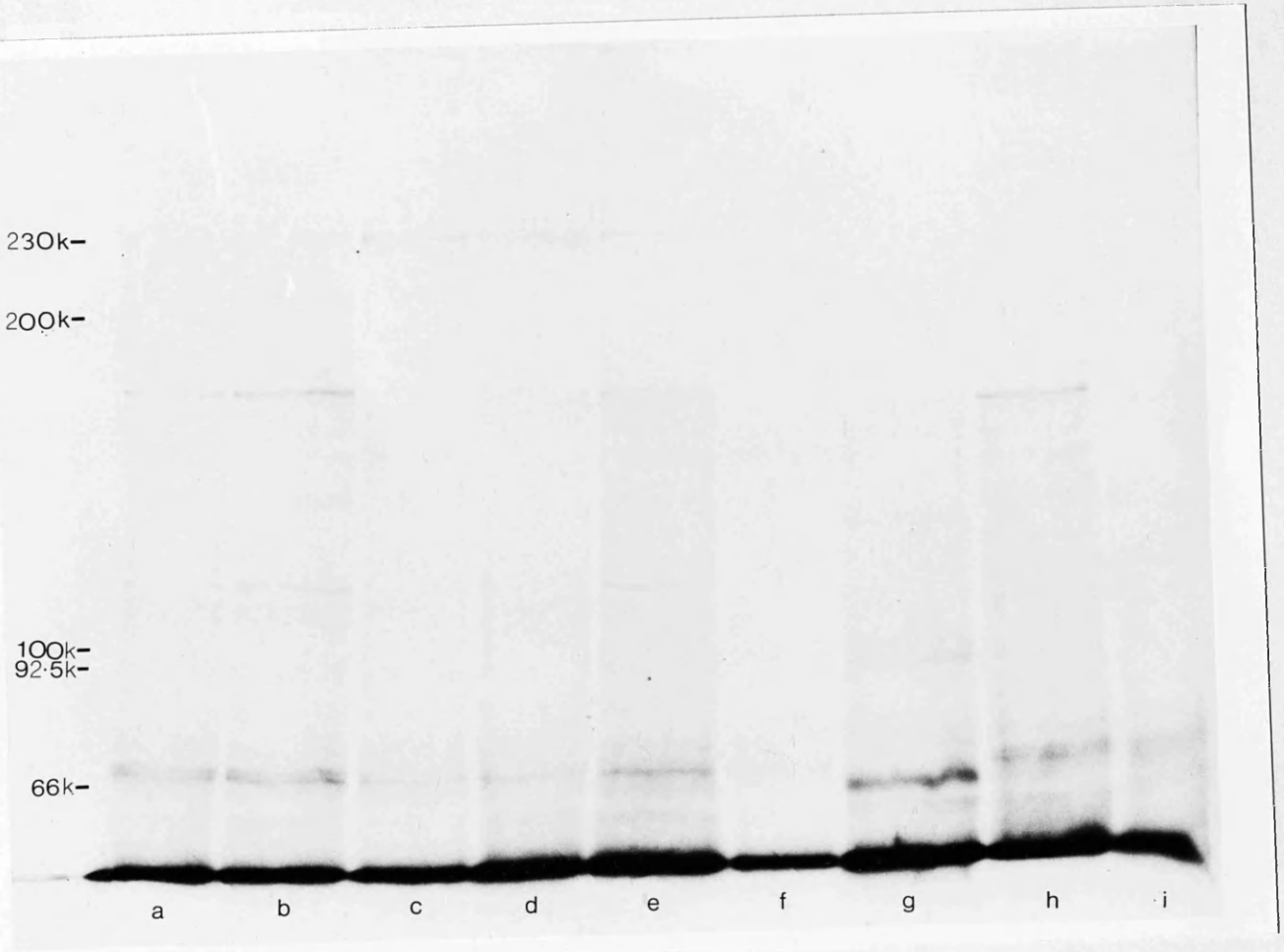

230k–
200k–
100k–
92·5k–
66k–
a
b
c
d
e
f
g
h
i

Plate 6.3 Immunoprecipitation of CR1 from Monocyte Lysates

CR1 was immunoprecipitated from the cell lysates of a monocyte culture which had been pulsed for 3 hours with no chase (tracks a, b and c). Track d represents the control precipitation with NMS. Samples were analysed on a 5% slab gel run under reducing conditions. A specific band of 225,000 daltons was seen in each of the lysates immunoprecipitated with monoclonal anti-CR1. No specific bands were seen in the control.

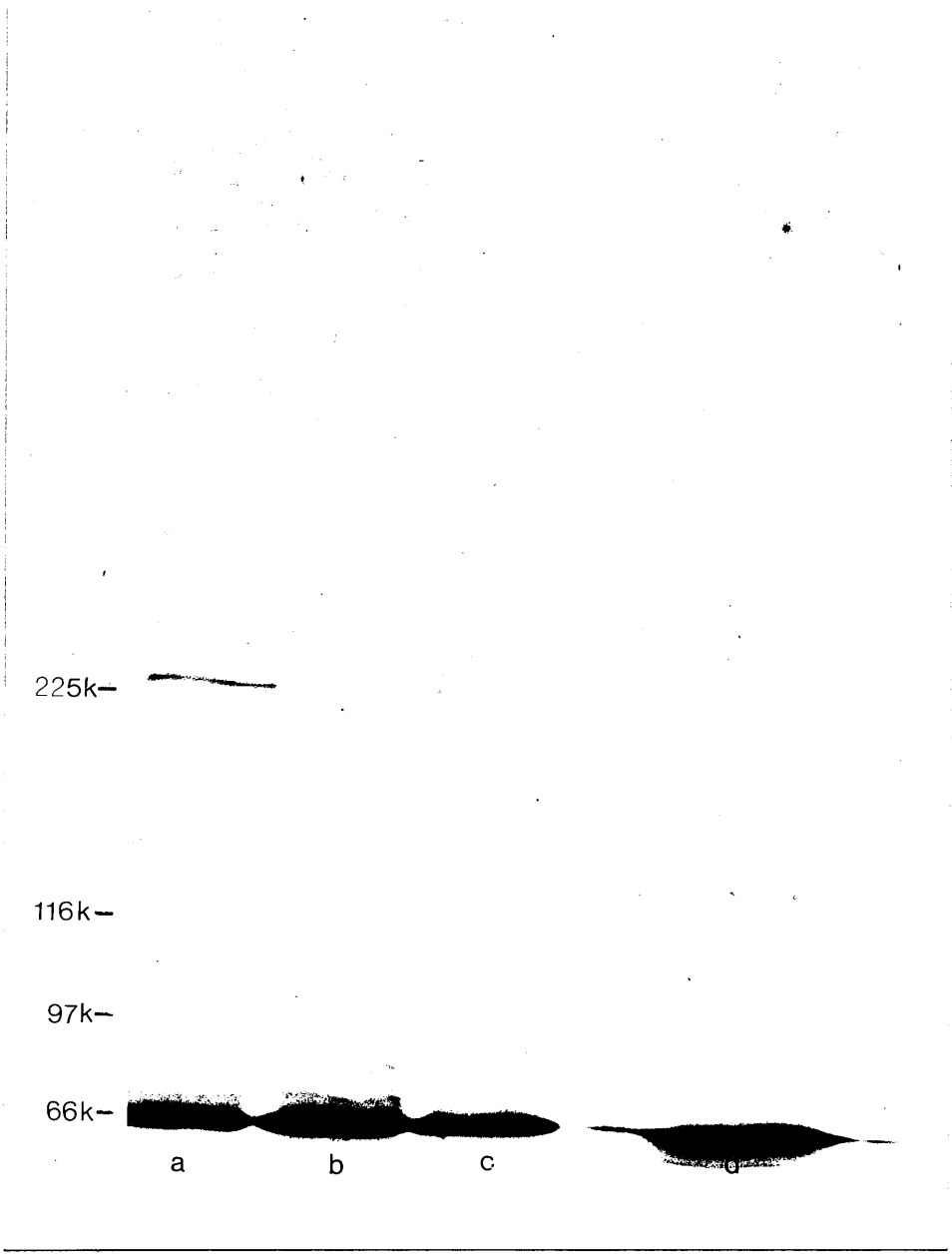
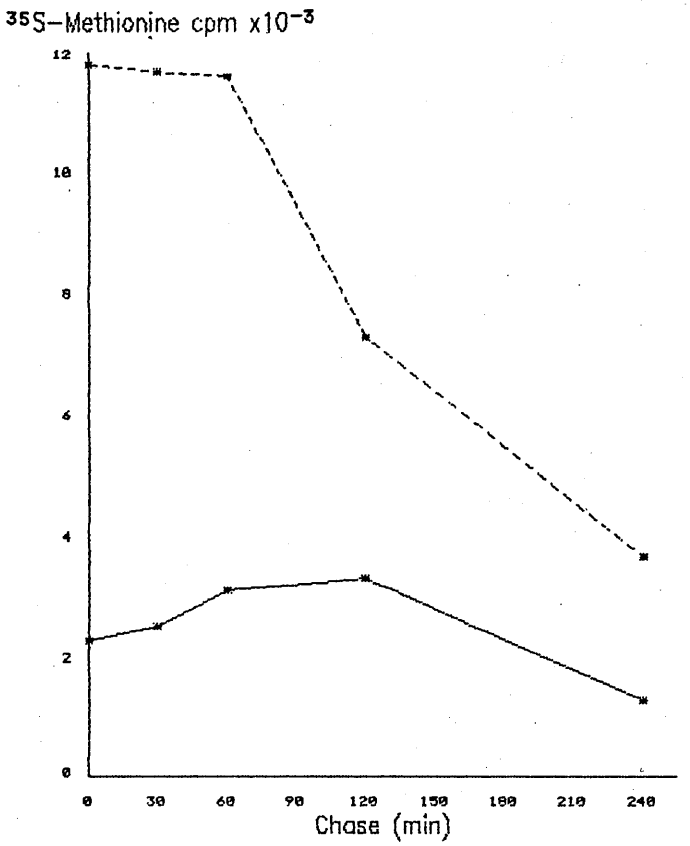

225k
116k
97k
66k
a
b
c
d

Fig 6.3 Mitogenic Stimulation of Lymphocytes

Legend

Lymphocytes which had been treated with PWM (0.5μg/1x$10^6$ cells) and unstimulated control lymphocytes were pulsed with $^{35}$S-methionine for 2 hours and chased for up to 240 min. The amount of $^{35}$S-methionine incorporation into the lymphocyte cell lysates was determined for the PWM stimulated lymphocytes (- - -) and for the control lymphocytes (_____). This number was then plotted against the chase time. The graph indicates that PWM stimulated lymphocytes can incorporate approximately 6-fold more $^{35}$S-methionine than control lymphocytes.


35S–Methionine cpm x10-3
12
10
8
6
4
2
0
0
30
60
90
120
150
180
210
240
Chase (min)

6.24 Pulse Chase Study in Lymphocytes

A lymphocyte culture which had been stimulated with PWM was pulsed for 2 hours and chased for 30, 60, 120 and 240 min on day 3 in culture. The lysates and supernatants were measured by TCA precipitation for incorporation of $^{35}$S-methionine. As with the monocytes (Fig 6.2), TCA precipitable protein in the lysate fell with increasing chase time, while those in the supernatant increased indicating that radiolabelled proteins were being secreted.

The lysates and supernatants were immunoprecipitated with anti-CR1 and the samples obtained were run reduced on a 7½% slab gel followed by fluorography. No specific band were detected in the lysates or supernatants (Plate 6.4). Numerous repeat pulse-chase experiments, with minor modifications in pulse and chase time were performed but no evidence of CR1 labelling was obtained.

6.25 Isolation of CR1 from Lymphocyte Lysates by Chromatography

Following the failure to isolate CR1 from lymphocyte lysates by immunoprecipitation it was decided to try to isolate the receptor using similar chromatographic procedures to those employed in the purification of CR1 (Fearon 1979). The method chosen was a modification of the one described by Dykman et al (1984) which had resulted in the successful isolation of CR1 from $^{125}$I-surface labelled PMN and monocytes.

The $^{35}$S-methionine labelled lysates from 14 separate lymphocyte culture tubes were put over a Biorex column which was then screened by measurement of TCA precipitable protein (Fig 6.4). The 2 elution fractions containing the most precipitable protein were pooled and then mixed with either C3-Sepharose or IgG-Sepharose.

The first 2 elution fractions from both the C3-Sepharose (Fig 6.5) and the IgG-Sepharose were subjected to SDS-PAGE on a 5% slab gel and fluorography. A faint band of approximately 210,000 daltons molecular weight was seen in the elution fractions from the C3-Sepharose. This band was not seen in the elution fractions from the IgG-Sepharose (Plate 6.5).

Plate 6.4 Immunoprecipitation of CR1 from Lymphocyte Lysates and Supernatants

Cell lysates and supernatants from a lymphocyte culture which had been pulsed for 2 hours with variable chase periods were immunoprecipitated with monoclonal anti-CR1. Tracks a to d represent the lysates chased for 30, 60, 120 and 240 min respectively. Tracks f to h represent the supernatants chased for 30, 60 and 120 min respectively. Track e is the lysate control and track i is the supernatant control which were immunoprecipitated with NMS. Samples were analysed on a 7½% slab gel run under reducing conditions. No specific bands were seen in any of the tracks.

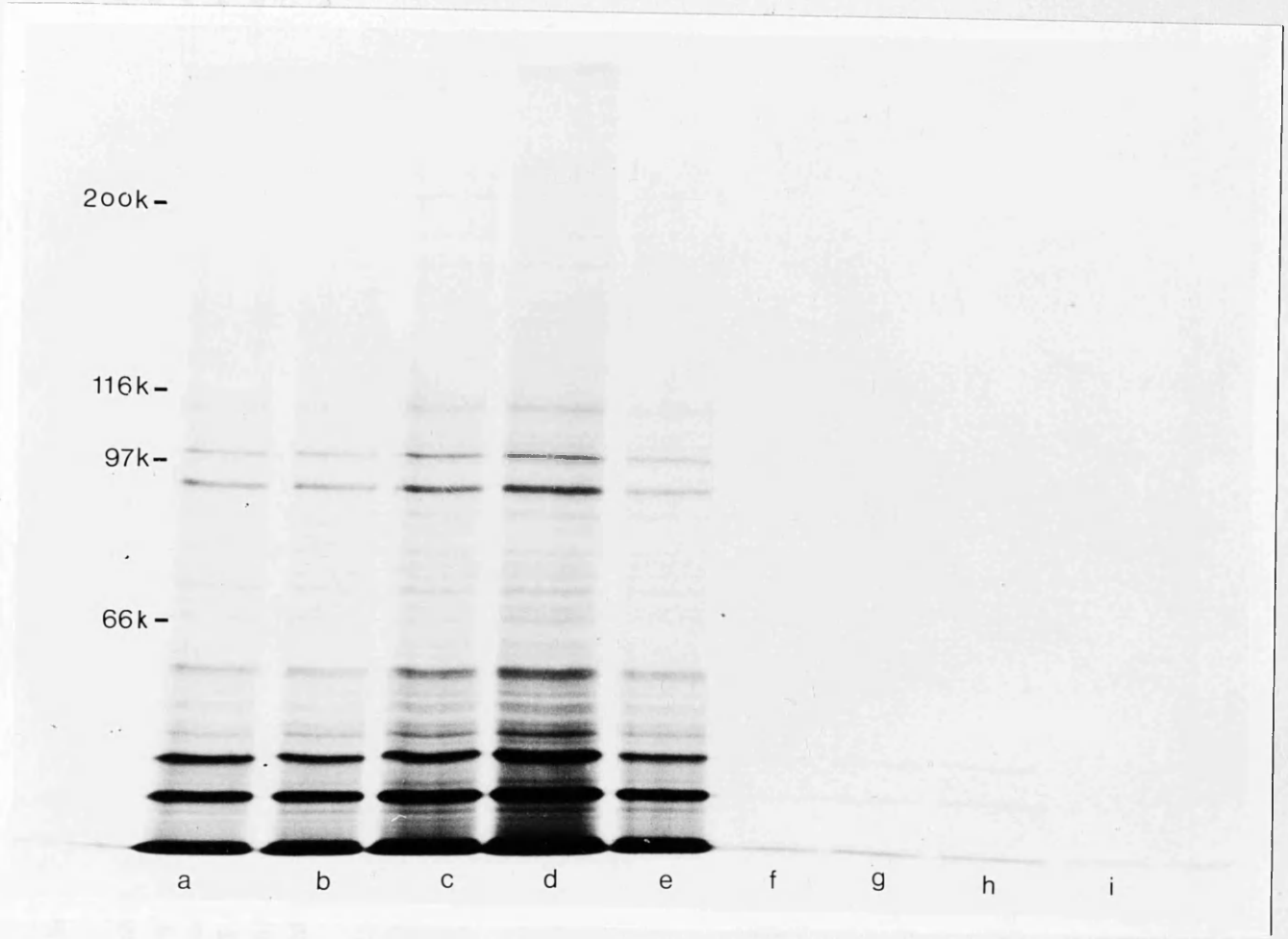

200k
116k
97k
66k
a
b
c
d
e
f
g
h
i

Fig 6.4 Biorex Column

Legend

Purification of CR1 from PWM stimulated lymphocytes (which had been pulsed with $^{35}$S-methionine for 2 hours and chased for 60 min), by cation exchange chromatography on a 1ml Biorex column. One millilitre fractions were collected and screened for CR1 by measurement of TCA precipitable protein. The number of $^{35}$S-methionine cpm in each fraction was then plotted against the fraction number. The arrow marked 1 on the X axis represents the application of the elution buffer. Fractions 9 and 10 were pooled as they contained the highest incorporation of $^{35}$S-methionine labelled protein.

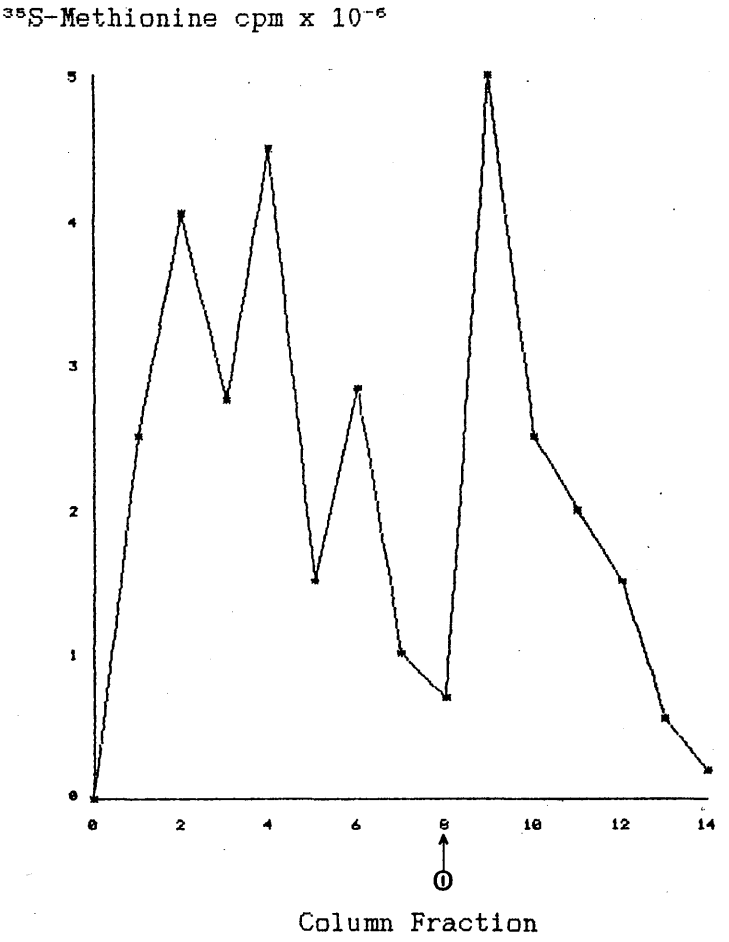

$^{35}$S-Methionine cpm x $10^{-6}$
5
4
3
2
1
0
0
2
4
6
8
10
12
14
0
Column Fraction

Fig 6.5 C3-Sepharose Affinity Chromatography

Legend

The pool from the Biorex column was absorbed with 25µl of a protein A cell suspension and then divided equally between 2 microcaps. To 1 of the microcaps 100µl of C3-Sepharose was added and to the other 100µl of IgG-Sepharose was added. After incubation for 2 hours bound protein was eluted by a series of 10 washes in elution buffer. The number of $^{35}S$-methionine incorporation into the eluted proteins was then plotted against the wash number.

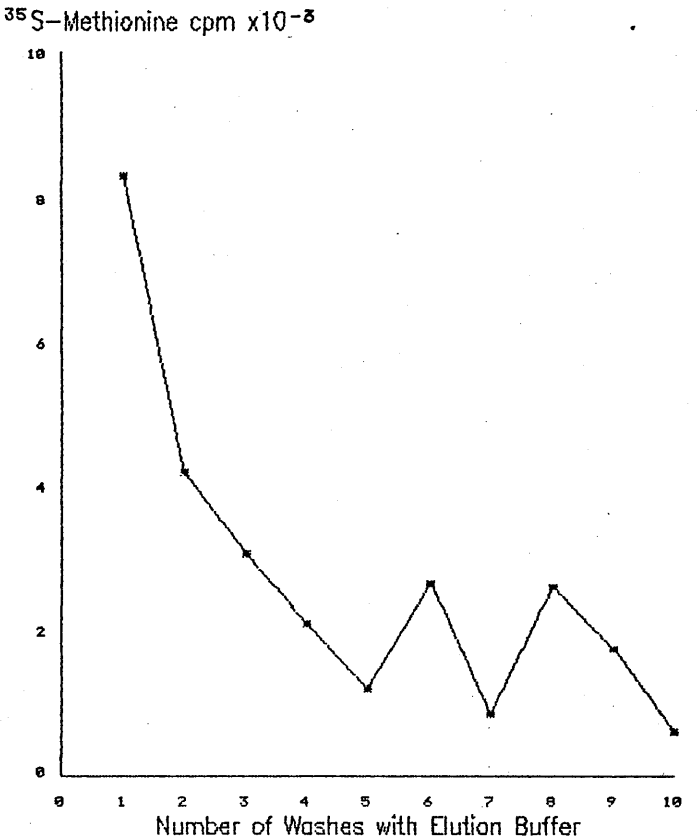
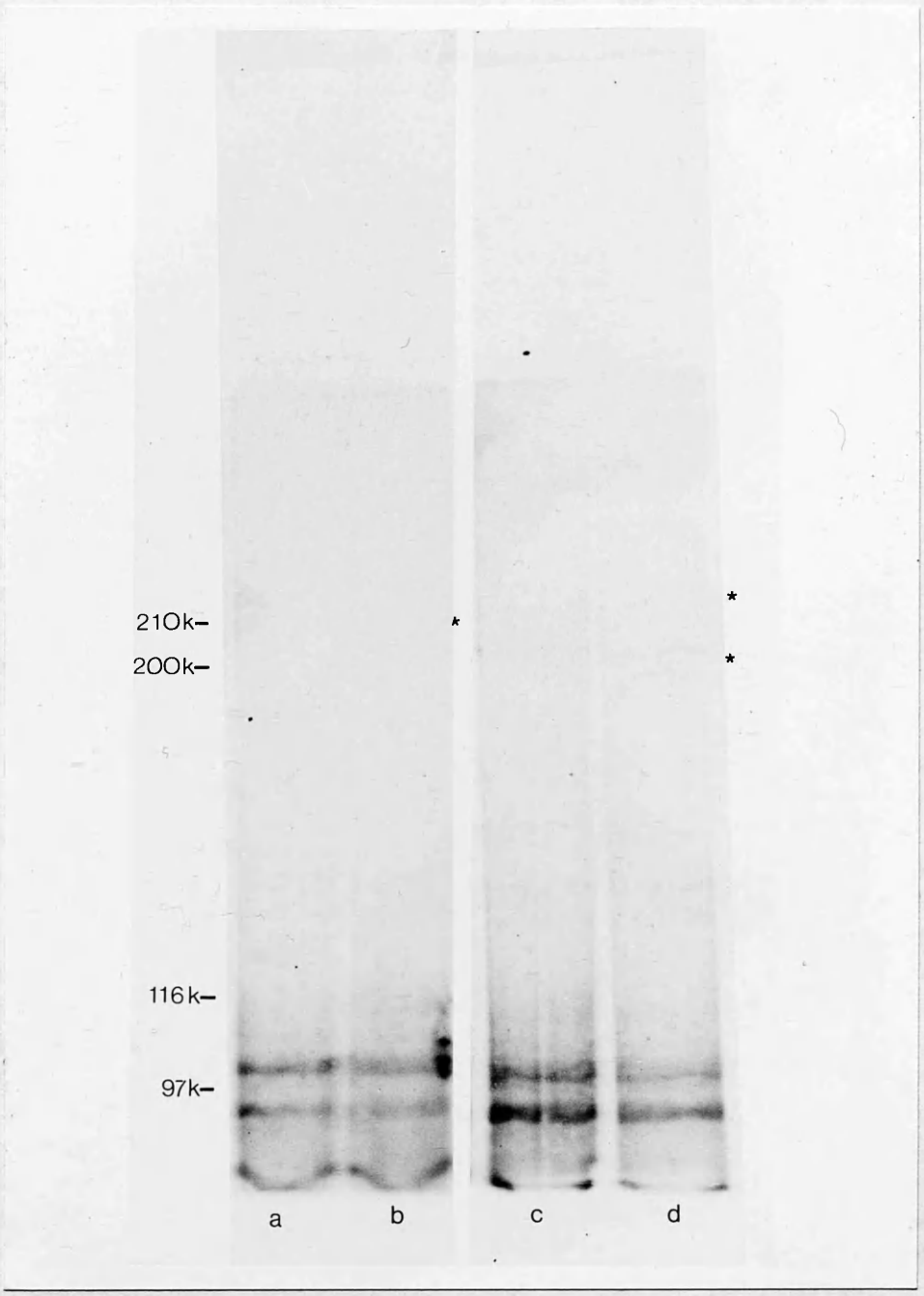

$^{35}$S–Methionine cpm x10$^{-3}$
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
10
Number of Washes with Elution Buffer

Plate 6.5 Chromatographic Purification of CR1 from Lymphocyte Lysates

CR1 was purified by chromatographic procedures from the cell lysates of a lymphocyte culture which had been pulsed for 2 hours and chased for 1 hour. Tracks a and b show the first and second elution fractions from the C3-Sepharose pellet while tracks c and d show the first and second elution fractions from the IgG-Sepharose pellet. Samples were analysed on a 5% slab gel run under reducing conditions. A very faint specific band with a molecular weight of 210,000 daltons can be seen in tracks a and b (the bands in track b is marked with a dot). This band was not seen in the elution fractions from the IgG-Sepharose pellet, although 2 very faint bands may be seen (these bands in track d are marked with a dot). The nature of these bands is unknown.


210k–
200k–
116k–
97k–
a
b
c
d

## 6.26 Discussion

The original intention of this study had been the comparison of CR1 synthesis in cultured monocytes and lymphocytes from normal individuals with the synthesis of CR1 in the same cells from patients with SLE. Before this could be attempted the conditions which would result in good incorporation of $^{35}$S-methionine into CR1 had to be established, as had the ability to immunoprecipitate CR1 from culture supernatants and cell lysates.

Monocytes were pulsed on day 7 of culture as C2 production (a measure of monocyte viability and ability to synthesis protein) is maximal at this time. Lymphocytes were pulsed on day 3 in culture after they had been stimulated with a concentration of PWM which was sufficient to produce maximum protein synthesis by the cells.

The immunoprecipitation method used was one which had resulted in the successful precipitation of CR1 from surface-labelled E using both a monoclonal and a polyclonal anti-CR1 antiserum (Plate 4.5). This method was also successfully utilised in the precipitation of C3 from $^{35}$S-methionine labelled monocyte supernatants (Plate 6.1). Thus the precipitation method was considered to be suitable for obtaining CR1 from cell lysates and culture supernatants.

CR1 was successfully immunoprecipitated from 2 of the 10 monocyte cell cultures set up (Plates 6.2 and 6.3). The molecular weights of these bands were 230,000 daltons and 225,000 daltons respectively. As previously discussed E CR1 expresses a size polymorphism. The most common of the E CR1 size variants has a molecular weight of 190,000 daltons when analysed under non-reducing conditions on SDS-PAGE. Analysis under reducing conditions increases the molecular weight of this band by 30,000 daltons. Dykman et al (1983b) showed that CR1 on the surface of monocytes had an identical molecular weight to that of the donors E, thus it may be that these bands represent the 190,000 daltons form of CR1. That the molecular weights of these bands are slightly higher than 190,000 daltons may be due to differences in the analytical systems used or they may indicate that there is a higher molecular weight intracellular form of CR1, which may result from the presence of a signal sequence which allows the receptor to become properly inserted into the membrane after which it is removed. To investigate this possibility more fully it would be necessary to compare the molecular weights of surface labelled monocyte CR1 with biosynthetically labelled monocyte CR1 to see if there was indeed a difference.

Atkinson and Jones (1984) studied the biosynthesis of CR1 in the HL-60 promyelocytic cell line and they have reported that there is an intracellular deglycosylated precursor form of CR1 with a molecular weight of 188,000 daltons which is 22,000 daltons smaller than the

surface CR1 form. The fact that these studies were performed on a cell line may account for the difference between this observation and my own.

No CR1 was immunoprecipitated from any of the monocyte culture supernatants. There is one report of a soluble form of CR1 (Yoon and Fearon 1985) but from the negative results of these experiments it was not possible to say if this could have arisen by secretion of CR1 from monocytes.

CR1 could not be immunoprecipitated from any of the 30 lymphocyte cultures using monoclonal or polyclonal anti-CR1 antisera. However, on one occasion when CR1 was isolated from lymphocyte cultures by cation exchange on Biorex and affinity chromatography on C3-Sepharose, a specific band with a molecular weight of 210,000 daltons was observed (Plate 6.5). In the non-reduced form this band would be equivalent to a band with a molecular weight of 180,000 daltons. A CR1 precursor (pro-CR1) can be immunoprecipitated from Epstein Barr virus transformed lymphocytes. The molecular weight of pro-CR1 was 9,000 daltons lower than the membrane-bound form of 190,000 daltons (Lublin et al 1985). Thus the band immunoprecipitated from the lymphocytes may be the proform of the 190,000 dalton CR1 variant. However, from the results of 1 culture it is not possible to make any conclusive statement.

This means of isolating lymphocyte CR1 was only attempted after the failure to immunoprecipitate the receptor by means of anti-CR1 antiserum. It proved to be a very time consuming procedure and to require the use of a large quantity of cells and other reagents. As time was limited it was not possible to continue this line of study although it may be that this method could be used to isolate CR1 from lymphocytes in future studies.

There are a number of explanations which may account for the inability to reproducibly immunoprecipitate CR1 from $^{35}$S-methionine labelled monocytes and lymphocytes. Firstly, synthesis of CR1 in these cell may normally proceed at a very low level, such that during the pulse period of 2 hours minimal amounts of CR1 were being synthesised. A low level of CR1 synthesis could arise if the monocytes and lymphocytes have internal reserves of CR1 as does the PMN (O'Shea et al 1985a). Sim and Sim (1983) were able to show that solubilised lymphocytes possessed more cofactor activity for the I mediated cleavage of C3b than did whole lymphocytes suggesting that lymphocytes have intracellular reserves of CR1 and Fearon and Collins (1983) reported that monocytes could up-regulate CR1 expression in response to chemotactic agents. Rapid synthesis of CR1 would not be required in the presence of large intracellular pools of the receptor. Thus in future studies it may be necessary to stimulate the monocytes with compounds which are known to increase the synthesis of CR1.

Secondly, CR1 may not contain enough methionine residues to allow sufficient incorporation of $^{35}$S-methionine into the molecule within the

short pulse time. Wong et al (1985) determined the amino acid composition of purified CR1 and found that methionine was the least abundant of the amino acids. Thirdly, Esparza, Fox and Schreiber (1986) showed that cell surface expression of CR1 on monocytes could be down-regulated by exposure of the cells to recombinant interferon γ. This down-regulation was considered to be due to shedding of the receptor as the total cellular levels of CR1 were also decreased. Sztein et al (1984) have shown that isolated human lymphocytes produce interferon γ constitutively. Thus if some lymphocytes were present as contaminants in the monocyte cultures they may have been producing sufficient quantities of interferon to cause down-regulation of CR1. This may have been a third contributory factor to low CR1 levels. This down-regulation of CR1 may have also applied in the lymphocyte cultures.

Although there was some degree of success in isolating CR1 from biosynthetically labelled monocytes and lymphocytes it was not a sufficiently reliable method to be considered suitable for the study of CR1 abnormalities in SLE patients. However I feel that this is an important area in which further research would be valuable as it may perhaps answer the question as to whether lower CR1 levels on these cells are caused by environmental factors or by a primary cellular defect.

Final Discussion

In the late 19th century it was noted that there seemed to be a protective substance in blood serum which could kill and lyse bacteria (Nuttal 1888; Buchner 1889). This protective substance was called complement (Ehrilch and Morgenroth 1906). In the first half of this century it was discovered that complement was a system comprising a number of proteins which interacted in sequence to cause lysis of sensitised E. In the latter half of this century research has shown that the complement system is comprised of at least 20 proteins which form the classical pathway, the alternative pathway, the terminal sequence and a group of control proteins. The end result of the activity of both the classical and alternative pathways is the cleavage of C3 which is the most abundant of the complement components being present in plasma at a concentration of 1-2mg/ml. This cleavage allows the assembly of the proteins of the terminal sequence into membrane attack complexes which result in the lysis of cells and invading microorganisms. In addition to this major function complement has a number of other biological activities (Table 1.1). One of the most important of these is the coordination of interactions between appropriate host cell types and pathogenic substances. This is achieved by means of complement receptors on the surface of host cells which can bind to fragments of complement proteins which are covalently attached to activators of complement.

Research into complement receptors was initiated in 1953 when Nelson (1953) described an adhesion phenomenon between human E and presensitised Treponema pallidium. As this adhesion was shown to be heat labile and destroyed by trypsin it was postulated that a receptor was responsible for the bond (Nelson and Nelson 1959). A few years later Nishioka and Linscott (1963) and Gigli and Nelson (1968) were able to demonstrate that C3 was the ligand responsible for immune adherence. This receptor has since become known as the immune adherence receptor, the C3b receptor or CR1. CR1 has now been shown to bind to a number of ligands apart from its major ligand C3b; these are: C4b, iC3, C3c and iC3b (Cooper 1969; Bokish and Sobel 1974; Ross and Polley 1975; Berger et al 1981; Medof and Nussenzweig 1984).

In the last few years much progress has been made in defining the receptor in molecular terms. The receptor was first isolated from E in 1979 and shown to be a large single chain polypeptide glycoprotein with a molecular weight of 205,000 daltons (Fearon 1979). There is now known to be a size polymorphism of CR1 with 4 separate allotypes having molecular weights of 190,000, 220,000, 250,000 and 160,000 daltons (Dykman et al 1983a and b; Wong et al 1983; Dykman et al 1984; 1985). This difference in size is not thought to be due to variations in the carbohydrate structure of the molecule (Wong et al 1983; Atkinson and Jones 1984; Dykman et al 1985). It may however be due to differences in the amino acid structure of the polypeptide chain. Klickstein et al (1985) have partially sequenced CR1 and shown that the polypeptide

chain consists of repeating homologous amino acid sequences. They therefore suggested that differences in size could be accounted for by addition or deletion of groups of repeat units of amino acids.

Although there are differences in size the functional capacities of the variants are similar (Seya et al 1985). These functions have been described in detail in the introduction. Briefly, CR1 isolated from E membranes has been shown in vitro to possess cofactor activity for the I mediated cleavage of soluble and surface-bound C4b and C3b to iC4b and iC3b respectively (Fearon 1979; Iida and Nussenzweig 1981). It may also act as a cofactor for the I mediated further cleavage of surface-bound iC3b to C3c and C3dg (Medof et al 1982) and substrate-bound C4b to C4c and C4d (Medof and Nussenzweig 1984) and it plays a role in the prevention of lysis of bystander cells by its ability to decay dissociate C4b2a3b and C3bBb formed on these cells (Fearon 1979; Fearon 1980). In vivo E CR1 is thought to play a role in transporting opsonised immune complexes from the circulation to the reticulo-endothelial system where they can be removed (Siegel et al 1981; Medof and Oger 1982; Cornacoff et al 1983; Jepson et al 1986; Sherwood and Virella 1986). The removal of complexes from the E is facilitated by the ability of CR1 to process the fixed C3b and C4b on the surface of the complexes to iC3b and C3dg and iC4b and C4d respectively. That this processing occurs is indicated by the observation that complexes which have been released from E show enhanced binding to CR2 bearing cells (Lam and Medof 1982). This capacity of E CR1 may therefore be of importance in the transfer of complexes from one cell type to another, as is the case where E transfer complexes to the cells of the liver during the passage of blood from the portal vein to the hepatic vein (Cornacoff et al 1983).

The primary function of CR1 on phagocytic cells is the binding of complexes opsonised with fragments of C3 and C4 degradation (Ehlenberger and Nussenzweig 1977). On stimulated phagocytes CR1 is also able to directly mediate phagocytosis of opsonised particles (Wright and Silverstein 1982; Pommier et al 1984; Changelian and Fearon 1985), and on unstimulated phagocytes CR1 can pinocytose small particles and soluble complexes into clathrin-coated pits (Fearon and Abrahamson 1983). This function of phagocytes may be important for the elimination of soluble immune complexes at sites of tissue inflammation.

Although the function of CR1 on B lymphocytes is still largely speculative it may play a role in the triggering of antigen-specific responses by binding to immune complexes containing C3 and activating B cell proliferation (Daha et al 1984).

CR1 on kidney podocytes may serve to prevent complement activation on the basement membrane of the glomerulus. It may also be able to directly mediate adsorbtive endocytosis of soluble immune complexes (Fischer et al 1986).

On nonphagocytic dendritic reticular cells CR1 may play a role in the retention of complement coated immune complexes in lymphoid follicles. (Klaus et al 1980) thus allowing the production of B memory cells (Klaus and Humphrey 1977).

Accordingly the main physiological functions of CR1 may be summarised as, (i) regulation of complement activation on adjacent cell surfaces, (ii) transport of immune complexes to suitable sites of removal, (iii) ingestion of opsonised particles and immune complexes and (iv) regulation of B lymphocyte function.

In the 4 years since this study was first initiated knowledge concerning the properties and functions of CR1 has considerably expanded. However one of the main issues to which this thesis addressed itself, that is, what is the cause of the reduced levels of E CR1 seen in patients with SLE, is still controversial. Originally the problem was deciding if the reduced levels were inherited or if they were acquired as a result of the disease process. Consequently one of the main aspects of this study was the comparison of CR1 expression on the E of identical and nonidentical twins in order to establish the relative roles of genetic and environmental factors in the regulation of E CR1 numbers in the normal population.

Prior to the twins study a polyclonal antiserum to CR1 was successfully raised. It was proposed to use this antiserum to measure E CR1 levels on the twins. However when the monoclonal antibody, E11, became available it was decided to use this instead of the polyclonal as one point determinations could be performed on the E. The conclusion from this study, which involved the measurement of E CR1 sites on 244 individuals (the largest number of individuals included in any E CR1 study thus far), was that genetic factors did not play a significant role in the regulation of CR1 expression on E. Thus CR1 numbers appeared to be regulated entirely by environmental factors. This finding is difficult to interpret in the light of recent findings by Wilson et al (1986a). In 1982 this group proposed a model which could account for the numerical polymorphism seen in the normal population. They showed that when CR1 sites on the E of 113 normal individuals were measured that the numbers appeared to fall into 3 groups, those with high numbers of E CR1, those with low numbers and those with intermediate numbers. This suggested that in the normal population there were 3 phenotypes, one for high (HH) CR1 expression, one for low (LL) CR1 expression and one for intermediate (HL) CR1 expression. The frequencies of these phenotypes in the normal population did not differ significantly from the frequencies predicted by the Hardy-Weinberg equilibrium for a 2 codominant allele model of inheritance and pedigree analysis of 7 families supported this conclusion. They went on to suggest that low levels of E CR1 expression seen in SLE patients were as a result of the prevalence of alleles determining low CR1 expression (Wilson et al 1982). In the past year this same group have published findings which lend further support to this 2 codominant allele model (Wilson et al 1986a). They have reported

the discovery of a restriction fragment length polymorphism of the CR1 structural gene which appears to correlate with low E CR1 expression. Individuals with low E CR1 expression have a 6.9 kb fragment, individuals with high CR1 expression have a 7.4 kb fragment and individuals with intermediate CR1 expression have both fragments. On analysis of their data it is difficult to ignore the evidence for the possession of only the 6.9 kb fragment resulting in low E CR1 expression. However the numerical expression of E CR1 in individuals with both fragments or individuals with only the 7.4 kb fragment was not quite so convincing as there was a considerable degree of overlap between the two groups. A possible reason for this overlap may be that CR1 numbers on individuals who are genetically predisposed to express high CR1 numbers on their E may have their E CR1 numbers reduced by environmental factors. In contrast individuals who express low CR1 numbers because of genetic factors, may have fairly constant CR1 numbers because they cannot be reduced significantly by environmental factors and they cannot be increased because of genetic control. This may explain the close correlation between the presence of the 6.9 kb fragment and low E CR1 numbers. If environmental influences were such that modulation of receptor numbers could occur easily in normal individuals this may be one explanation for the observation that no genetic influence could be demonstrated on E CR1 expression in twins. To test this hypothesis it may be interesting to study the influence of genetic and environmental factors on the expression of E CR1 only on those twins who have low CR1 numbers since these receptors may be less susceptible to modulation by environmental factors.

Having shown that environmental factors play a role in the regulation of E CR1 numbers in the normal population, it was decided to investigate possible environmental factors which could modulate CR1 expression on normal E in an attempt to determine if these same factors could contribute towards the low numerical expression of CR1 seen on the E of patients with SLE.

At the present moment the most likely explanation for reduced CR1 levels on the E of patients with SLE is the proteolytic stripping of CR1 from E as the E carrying immune complexes come into contact with the protease rich reticulo-endothelial cells. Thus the ability of a selection of proteolytic enzymes to remove E CR1 was investigated. It was found that at physiological concentrations none of the enzymes tested had the capacity to decrease E CR1 (Table 4.5). At high concentrations, trypsin and elastase could significantly decrease CR1 sites (Table 4.5). However it was considered unlikely that these concentrations of proteases would be present in the plasma and in any case the plasma contains a variety of protease inhibitors which would rapidly inactivate these proteases. It could not however be ruled out that E CR1 could be removed when the cells come into close contact with the proteinase rich phagocytes during transfer of immune complexes in the liver and spleen.

The blockade of E CR1 sites by immune complexes is considered by most to be a less likely explanation for reduced E CR1 levels (Wilson et al 1982; Minota et al 1983; Ross et al 1985). However experiments performed in this thesis would seem to indicate that such a mechanism may indeed result in the inability to detect E CR1. In vitro it was shown that E CR1 could be blocked by large opsonised IgG aggregates (Table 4.7) and in vivo it was shown that E CR1 expression on 6 individuals was reduced by the consumption of 1.2 litres of cows milk, a rich source of food antigens (Fig 4,6; Table 4.8). This latter experiment in particular indicated how easily CR1 expression could be modified by environmental influences. Further experiments in this particular aspect might be of value in deciding if blockade of E CR1 does truly constitute the mechanism of reduced CR1 levels on the E of SLE patients.

Although it has been known for a number of years that patients with SLE have low CR1 levels, at the start of this study it was not known if CR1 was reduced on other cell types. One of the aims of this study was the measurement of CR1 on the PMN of normal individuals and on patients with SLE in order to determine if both cell types in these patients had reduced levels of CR1 expression in comparison to normal. It was shown that PMN from normal individuals expressed 3 distinct pools of PMN CR1. One of these pools was expressed at 0°C, a second was expressed after warming the cells to 37°C and the third was expressed in response to stimulation with the synthetic chemotactic agent FMLP. Expression of the pool at 37°C was not dependent upon protein synthesis, an intact cytoskeleton or energy while the pool expressed in response to FMLP was dependent upon an intact cytoskeleton and energy but did not require de novo protein synthesis.

All 3 of these pools were found to be reduced in patients with active SLE but were normal in those with inactive SLE (Fig 5.12). The most severely depleted pool was that which was expressed in response to stimulation with FMLP. If this mechanism of increasing CR1 expression in response to chemotactic stimuli released at sites of tissue inflammation is defective then it may indicate that the recognition of and phagocytic function for pathological particles by those PMN may be impaired. If this were so it would contribute to the tissue deposits of immune complexes seen in SLE.

The mechanism whereby this reduction is brought about is unknown. As in the study of reduced levels of E CR1 expression in SLE the question arises whether reduced levels result from a genetic defect or are caused by the disease process. PMN show a numerical polymorphism in CR1 expression with numbers varying widely among individuals. These numbers do not appear to fall into distinct groups of individuals having high, medium or low CR1 expression. Wilson et al (1986a) reported that the presence of restriction fragments (6.9 kb and 7.4 kb) did not influence expression of CR1 on neutrophils as they did on E although these fragments are linked to the same structural gene which controls the structure of both E and PMN CR1. Thus there is no evidence as yet to

suggest that PMN CR1 expression is genetically controlled in the normal population. The results from my studies do however suggest that in patients with SLE CR1 expression on PMN can be modulated by factors associated with the disease as it was found that the number of PMN CR1 correlated significantly with the number of E CR1 in SLE patients (Fig 5.13 b, c and d) and both changed in parallel with disease activity (Table 5.7). In addition it was shown that SLE patients who were in an inactive phase of disease had levels of PMN CR1 expression which did not differ significantly from normal (Fig 5.12). This suggests that the decrease in PMN CR1 number is an acquired defect. The precise mechanism whereby this occurs is outwith the scope of this thesis however, it must merit further investigation.

It may be possible that loss of the receptor occurs after binding to opsonised immune complexes bearing C3 fragments. It has been shown by a number of groups that PMN can internalise CR1 bound to its ligand by the process of endocytosis ((Fearon et al 1981; Abrahamson and Fearon 1983; Hogg et al 1984). Abrahamson and Fearon (1983) showed that once the receptor-ligand complexes had been taken into clathrin-coated vesicles they were transported to azurophile granules where they were degraded. Thus the receptors were not recycled. This might imply that as the immune complex load is greater in SLE patients more receptors are internalised and degraded. Thus there is an increased demand for receptors to be brought to the surface from internal pools and therefore both surface expression of CR1 and internal pools of CR1 eventually become depleted. As the disease becomes inactive less complexes are available for clearance and so the demand for receptors is reduced and expression of PMN CR1 can return to normal.

However Taylor et al (1983b) has reported that there was no significant internalisation of soluble immune complexes which contained dsDNA and anti-dsDNA from SLE patients, after they had bound to PMN through CR1. It may therefore be possible that internalisation of CR1-ligand complexes in SLE patients occurs not by endocytosis but by phagocytosis. PMN stimulated with FMLP and soluble fibronectin are able to phagocytose particles by means of CR1 (Pommier et al 1984). As chemotactic factors are released at sites of tissue inflammation and soluble fibronectin is also present PMN may become phagocytically activated and internalise complexes with the same resultant depletion of internal pools. Taylor et al (1983b) may not have observed internalisation of complexes because the conditions were not appropriate for activation of PMN CR1.

An alternative explanation for the depletion of PMN CR1 expression is that there is a defect in the ability of the cell to synthesise CR1 as a result of a primary abnormality in the CR1 gene or in its expression. To investigate this possibility monocyte and lymphocyte cultures were pulsed with $^{35}S$-methionine with the aim of immunoprecipitating CR1 from cell lysates and supernatants. Both of these cell types have been shown to be defective in CR1 function or CR1 expression in SLE patients. Hurst et al (1984) reported a significant reduction in the rate of complement

mediated phagocytosis by blood monocytes in patients with SLE. This reduction was most evident in patients with active disease and Wilson et al (1986b) reported that B cells from 17 SLE patients had significantly reduced CR1 expression in comparison to normal individuals.

CR1 was successfully immunoprecipitated from 2 normal monocyte culture lysates (Plate 6.2 and Plate 6.3) and was isolated from 1 normal lymphocyte culture lysate by affinity chromatography on C3-Sepharose (Plate 6.5). For reasons discussed in chapter 6.26 it was not considered to be a viable method for studying the biosynthesis of CR1 in patients with SLE. This is unfortunate as the ability to do so may provide a valuable insight into the mechanism of reduced CR1 numbers on peripheral blood leukocytes

In conclusion therefore the different aims of this study have been achieved though in varying degrees. Although 4 years have passed since the start of this investigation the questions which were raised at the beginning are still extremely topical. Thus far no one has been able to study the biosynthesis of CR1 in normal leukocytes nor in leukocytes from patients with SLE. Therefore the question still remains unanswered as to whether there is a defect in the biosynthesis of CR1 in SLE patients. The few results obtained from the biosynthetic study in chapter 6 show that it is possible to immunoprecipitate CR1 from normal cells and perhaps studies in the future should continue to look at this aspect.

Although in the past year one group has published a report on the reduction of CR1 on the neutrophils of SLE patients no one has defined the pools of CR1 which exist in normal PMN. In this thesis I have been able to show that there are 3 pools of CR1 which all have different requirements for their expression. In addition I have shown that there is a numerical polymorphism of CR1 on PMN and that the numbers of CR1 on PMN of patients with SLE are severely depleted. This depletion seems to correlate with disease activity. The cause of this depletion still remains to be investigated.

In conclusion therfore we have been unable to find genetic control of E CR1 expression in normal individuals. However it is quite possible that regulation of the expression of the CR1 structural gene is under genetic control and at some time in the life span of the E the CR1 genotype reflects the phenotype, but at other times as environmental factors have a significant influence on E CR1 numbers the phenotype is altered. It may be that the suseptibility of the phenotype to environmental factors is exaggerated on the E of patients with SLE where levels of CR1 can be reduced dramatically during periods of disease activity and return to normal when the patient is in an inactive phase of disease (Ross et al 1985; Holme et al 1986). The factors which reduce E CR1 numbers have still not been characterised and must therefore merit further investigation.

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